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BioReD: Biomarkers and Tools for Reductive Dechlorination Site Assessment, Monitoring and Management

SERDP Project ER-1586

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14. ABSTRACT
Reductive dechlorination plays a major role in the transformation and detoxification of chloroorganic pollutants, including chlorinated ethenes, and the application of molecular biological tools (MBTs) has already impacted site assessment and bioremediation monitoring at many DoD sites. Unfortunately, limitations in current tools provide an incomplete picture of the reductively dechlorinating bacterial community, thus limiting the value of the analysis. To overcome the current limitations and more accurately assess, predict, monitor and manage reductive dechlorination processes at contaminated DoD sites, this research effort identified novel reductive dechlorination biomarker genes and developed MBTs and approaches that improve our understanding of target gene presence, abundance, and expression, and thus, contaminant detoxification.

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Executive Summary

Reductive dechlorination plays a major role in the transformation and detoxification of chloroorganic pollutants, including chlorinated ethenes, and the application of molecular biological tools (MBTs) has become a valuable step in site assessment and bioremediation monitoring at many Department of Defense (DoD) sites. Unfortunately, the current tools have limitations and provide an incomplete picture of the reductively dechlorinating microbial community. A SERDP Expert Panel Workshop on MBTs, academics and DoD remediation project managers (RPMs) stressed the potential value of the technology to address DoD mission needs but also emphasized shortcomings. To overcome the current limitations and more accurately assess, predict, monitor and manage reductive dechlorination processes at contaminated DoD sites, novel reductive dechlorination biomarker genes were identified and refined MBT approaches, in particular quantitative real-time PCR (qPCR), were tested and validated. These results improve our understanding of how target gene presence, abundance, and activity correlate with the reductive dechlorination of chlorinated solvents to environmentally benign end products.

This work contributed to building a more comprehensive suite of nucleic acid-based tools for site assessment and bioremediation monitoring, along with new protocols for tools application and results interpretation, to generate confidence in MBT application and achieve widespread acceptance by regulators and RPMs. Further, experiments characterizing dechlorinating cultures and reductive dehalogenase (RDase) enzyme systems advanced understanding of the reductive dechlorination process by elucidating interferences with, and inhibitory effects of, chlorinated ethanes and chlorinated methanes on the degradation of chlorinated ethenes.

The application of advanced MBTs will allow for more efficient allocation of resources to sites amenable to bioremediation technologies, promote science-driven site management, endorse widespread implementation of Monitored Natural Attenuation (MNA) and enhanced bioremediation approaches, facilitate regulatory acceptance,

promote site closures, and ultimately provide significant costsavings to the DoD. The improved tools and the deeper scientific understanding of the reductive dechlorination process form a basis for further advances, so that MBTs can be applied with confidence to a broad range of sites with different specific contamination challenges, including large dilute plumes, fractured matrices, DNAPL source zones, and mixed contaminant plumes.

This SERDP research project has resulted in a series of deliverables including 37 peer-reviewed manuscripts, 5 peer-reviewed book chapters, 13 student theses, over 100 oral presentations including several instructional workshops and webinars, and 87 poster presentations. Another deliverable is an Excel spreadsheet for calculating chlorinated solvent partitioning between the aqueous phase, the headspace and an organic phase (i.e., NAPL) in closed vessels. This partitioning calculator can be found on the Löffler Lab web site (http://web.utk.edu/~microlab/LoefflerLab/Projects.html). Many deliverables were presented to wide audiences of practitioners at applied conferences. Several of the students trained in this project pursued employment in environmental consulting. Taken together, this research effort advanced scientific understanding of the reductive dechlorination process, contributed to translating knowledge into practice, and will have a lasting impact of the environmental engineering profession.

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List of Acronyms

1,1-DCA	1,1-Dichloroethane
1,1,1-TCA	1,1,1-Trichloroethane
1,1,2-TCA	1,1,2-Trichloroethane
1,1,2,2-TeCA	1,1,2,2-Tetrachloroethane
1,1-DCP	1,1-Dichloropropane
1,2-DiBr-3-CP	1,2-Dichloropropane
1,3-DCP	1,3-Dichloropropane
1,2-DCP	1,2-Dichloropropane
1,2-DCA	1,2-Dichloroethane
1,2,3-TCP	1,2,3-Trichloropropane
1-CP	1-Chloropropane
2-CP	2-Chloropropane
2,2-DCP	2,2-Dichloropropane
2-Br-1-CP	1-Bromo-1-chloropropane
6-FAM	6-Carboxyfluorescein
BES	2-Bromoethanesulfonate
BioReD	Biomarkers for reductive dechlorination
BSA	Bovine serum albumin
BTEX	Benzene, toluene, ethylbenzene, xylenes
CA	Monochloroethane
CARD-FISH	Catalyzed reporter deposition fluorescence *in situ* hybridization
cDCE	*cis*-1,2-Dichloroethene
CF	Chloroform
CP	Chloropropane
CSIA	Compound-specific stable isotope analysis
CT	Carbon tetrachloride
DCA	Dichloroethane
DCM	Dichloromethane
Dhb	*Dehalobacter*
Dhc	*Dehalococcoides*

Dhgm	*Dehalogenimonas*
DNQ	Detectable but not quantifiable
FISH	Fluorescence *in situ* hybridization
FOI	Frequency of incorporation
GC	Gas chromatograph
Cy	Fluorescent cyanine dye
ITRC	Interstate Technology Research Council
JOE	6-Carboxy-4', 5'-dichloro-2', 7'- dimethoxyfluorescein
MEGAN	MEtaGenomeANalyzer
MTBE	Methyl-*tert*-butyl ether
NAPL	Non-aqueous phase liquid
NCBI	National Center for Biotechnology Information
PCBs	Polychlorinated biphenyls
PCE	Tetrachloroethene
qPCR	Quantitative real-time PCR
Rif	Rifampicin
RPM	Remediation project manager
RDase	Reductive dehalogenase
SDS	Sodium dodecyl sulfate
SSC	Saline-Sodium Citrate (SSC) buffer
TAMRA	6-Carboxy-tetramethylrhodamine
TCB	Trichlorobenzene
TCE	Trichloroethene
TCP	Trichloropropane
tDCE	*trans*-1,2-Dichloroethene
VC	Vinyl chloride (monochloroethene)
VOC	Volatile organic compound

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Abstract

Background. Reductive dechlorination plays a major role in the transformation and detoxification of chloroorganic pollutants, including chlorinated ethenes, and the application of molecular biological tools (MBTs) has already impacted site assessment and bioremediation monitoring at many DoD sites. Unfortunately, limitations in current tools provide an incomplete picture of the reductively dechlorinating bacterial community, thus limiting the value of the analysis. To overcome the current limitations and more accurately assess, predict, monitor and manage reductive dechlorination processes at contaminated DoD sites, this research effort identified novel reductive dechlorination biomarker genes and developed MBTs and approaches that improve our understanding of target gene presence, abundance, and expression, and thus, contaminant detoxification.

Objectives: The specific research objectives focused on (i) elucidating co-contaminant effects on the reductive dechlorination of chlorinated ethenes, (ii) applying microarrays as tools to discover novel reductive dechlorination biomarker genes, (iii) developing specific, sensitive and economic quantitative real-time PCR (qPCR) assays for the enumeration of the most promising biomarker genes and their transcripts, (iv) evaluating whether biomarker transcript quantification and transcript-to-gene abundance ratios correlate with reductive dechlorination activity; (v) testing fluorescence *in situ* hybridization (FISH) approaches for visualizing relevant microorganisms, and (vi) applying the new biomarkers and procedures to field samples.

Technical Approach: Several reductively dechlorinating consortia maintained with chlorinated ethenes, chlorinated ethanes and chlorinated methanes as electron acceptors provided biomass for whole cell and cell-free extract enzyme assays to assess inhibition caused by other chlorinated solvents. Three complementary microarray approaches identified reductive dechlorination biomarker genes, for which quantitative real-time PCR assays were designed and validated. *Dehalococcoides* pure cultures and *Dehalococcoides*-containing consortia were used to interrogate three complementary microarrays systems to elucidate genes that could serve as reductive dechloirnation biomarkers.

Results: SERDP Project ER-1586 i) elucidated the underpinning mechanisms of reciprocal inhibition of cDCE and VC reductive dechlorination by CF and 1,1,1-TCA, ii) determined inhibition constants (K_i values) helpful to predict if co-contaminant inhibition will occur, iii) demonstrated that culture blends can relieve inhibition of cDCE and/or VC reductive dechlorination caused by co-contaminants such as CF and 1,1,1-TCA; iv) identified new biomarker genes encoding specific RDases including *cfrA* [CF to DCM and 1,1,1-TCA to 1,1-DCA], *dcrA* [1,1-DCA to CA], and *dcpA* [1,2-DCP to propene], v) designed and validated qPCR assays for *Dhc* biomarker gene (and transcript) quantification, vi) validated that Sterivex cartridges are useful for on site biomass collection (in collaboration with SERDP Project 1561), vii) confirmed that the new qPCR tools can be applied to biomass collected from contaminated site samples, and viii) demonstrated that *Dhc*-to-total bacteria 16S rRNA gene ratios greater than 0.0005 (0.05%) correlate with ethene formation (i.e., detoxification).

Benefits: A broader suite of reductive dechlorination biomarkers and enhanced tools for quantitative enumeration allows for a more efficient allocation of resources to sites amenable to bioremediation technologies, promote science-based site management decisions, endorse widespread implementation of Monitored Natural Attenuation (MNA) and enhanced bioremediation, facilitate regulatory acceptance, promote site closures, and ultimately provide significant financial payback to the DoD. Further, the tools and knowledge generated benefit the scientific community exploring the distribution and ecology of reductively dechlorinating organisms, unravel the specific requirements of keystone reductively dechlorinators (e.g., *Dehalococcoides*), and shed light on the relevance of lateral gene transfer for the dissemination of reductive dehalogenase genes.

Objectives

The BioReD project aims were to identify novel biomarker genes involved in the reductive dechlorination of chlorinated solvents relevant to DoD sites, and to develop approaches that improve our understanding of biomarker gene presence, abundance, and activity. The specific research objectives focused on (i) elucidating the effects of co-contaminant interactions on the reductive dechlorination of chlorinated ethenes, (ii) applying microarrays as tools to discover novel reductive dechlorination biomarker genes and processes, (iii) developing specific, sensitive and economic quantitative real-time PCR (qPCR) assays for the enumeration of the most promising biomarker genes and their transcripts, (iv) evaluating whether biomarker transcript quantification and transcript-to-gene abundance ratios correlate with reductive dechlorination activity; (v) testing fluorescence *in situ* hybridization (FISH) approaches for visualizing relevant microorganisms, and (vi) applying the new biomarkers and procedures to field samples.
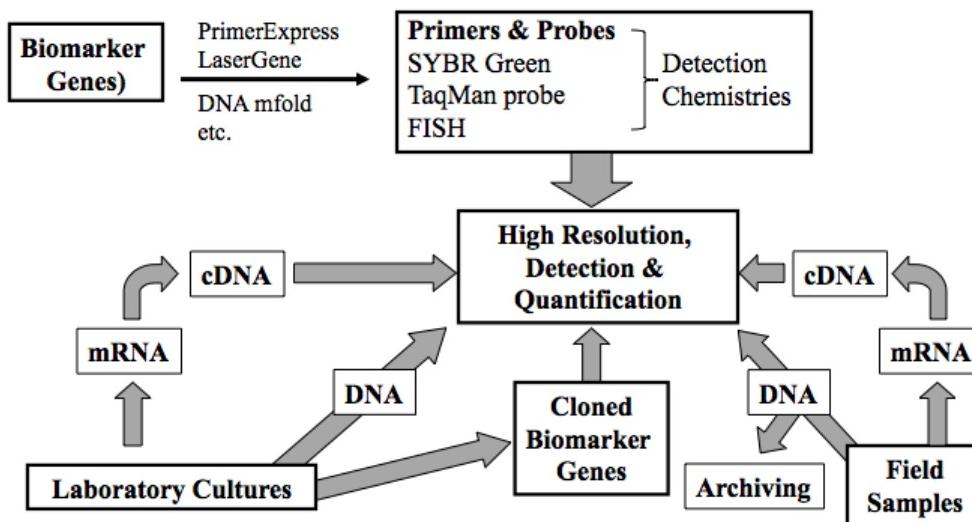
Schematic illustration of the approach applied for biomarker gene identification.

Background

Research over the past two decades has significantly advanced understanding of the microbiology contributing to the transformation of chlorinated ethenes under anoxic conditions. Specialized bacteria use chlorinated compounds as electron acceptors to

conserve energy from reductive dechlorination reactions in a process called organohalide respiration. Foremost among the prominent bacterial populations involved in the reductive dechlorination process are strains of the species *Dehalococcoides mccartyi* (*Dhc*) (87). *Dhc* were identified as key players in promoting the complete reductive dechlorination of chlorinated ethenes to environmentally benign ethene, and a link between the presence of *Dhc* and detoxification (i.e., formation of ethene and inorganic chloride) was established (57). Bacteria belonging to a variety of bacterial genera, including *Dehalogenimonas* (*Dhgm*) (108), *Dehalobacter* (*Dhb*) (44, 46, 60, 75), *Sulfurospirillum* (94, 130, 131), *Desulfitobacterium* (35, 36, 100, 107, 149), *Desulfuromonas* (77, 140), and *Geobacter* (10, 138), also perform organohalide respiration, and contribute to the reductive transformation of chlorinated solvents, including chlorinated ethenes (e.g., PCE$\rightarrow$TCE; TCE$\rightarrow$cDCE) chlorinated ethanes (e.g., 1,1,1-TCA$\rightarrow$CA; 1,1,2-TCA$\rightarrow$VC; 1,2-DCA$\rightarrow$VC), chlorinated methanes (e.g., CF$\rightarrow$DCM$\rightarrow$CO$_2$) and chlorinated propanes (e.g., 1,2-DCP$\rightarrow$propene).

Successful bioremediation applications at sites impacted with chlorinated ethenes take advantage of the microbial reductive dechlorination (i.e., organohalide respiration) process. The application of molecular biological tools (MBTs) for *Dhc* biomarker quantification has proven useful for site assessment and bioremediation monitoring. A SERDP/ESTCP-sponsored workshop on *"Research and development needs for the environmental remediation application of molecular biological tools"* concluded that the lack of sufficient biomarker targets hampers site assessment and efficient site management. Additional reductive dechlorination biomarkers are needed to improve prognostic and diagnostic monitoring of the dechlorinating bacterial populations and their activities of interest. A more comprehensive suite of reductive dechlorination biomarkers will support prognostic site assessment and determine whether biostimulation alone can achieve remediation goals or if bioaugmentation is necessary. Further, a refined tool set will advance bioremediation monitoring programs and inform remediation project managers (RPMs) about strategies to adjust bioremediation performance to efficiently achieve remedial goals.

Task 1: Substrate Range and Substrate Interactions

Spreadsheet for Calculating Partitioning of Chlorinated Solvents and Daughter Products Between the Aqueous and Gas Phases in Closed Vessels

Retrieving the physical properties (i.e., density, aqueous solubility, Henry's law constants, molecular weight) to calculate the appropriate amounts of each compound required for the different cultures is time-consuming and tedious. We completed the design of an Excel spreadsheet that conveniently provides all of this information. This spreadsheet automatically calculates compound partitioning between aqueous phase and headspace in vessels of different sizes and different aqueous phase-to-headspace volume ratios. For compounds with low water solubility, the spreadsheet will determine if NAPL is present and calculate the NAPL volume, the amount of compound (in mmoles) present in the NAPL phase, and the percent of the total contaminant mass present in the NAPL phase. When NAPL is present, the aqueous phase concentration is assumed to be at the maximum solubility (C_{sat}). The spreadsheet calculates the partitioning for up to three individual vessels (i.e., vessel 1, 2, or 3) with different volumes (aqueous and headspace) and different amounts of the chlorinated compound. As an example, the spreadsheet for calculating the phase partitioning of PCE is shown in Figure 1.1.

The current version of the spreadsheet contains information for chlorinated ethenes (PCE TCE, cDCE, tDCE, 1,1-DCE, VC), chlorinated methanes (CT, CF, DCM, CM), chlorinated propanes (1-CP, 2-CP, 1,1-DCP, 1,2-DCP, 1,3-DCP, 2,2-DCP, 1,2,3-TCP, 2-Br-1-CP, 1,2-DiBr-3-CP), chlorinated ethanes (1,1,2,2-TCA, 1,1,1-TCA, 1,1,2-TCA, 1,1-DCA, 1,2-DCA, CA) and relevant non-chlorinated alkanes and alkenes (ethene, ethane, methane, propene). This spreadsheet is available to the community via Dr. Löffler's University of Tennessee website: http://web.utk.edu/~microlab/LoefflerLab/Projects.html

Compounds	H$_2$O volume (ml)	compound (µl)	headspace (ml)	carrier phase (ml)	sediment (g)	sediment f$_{om}$
	rtitioning in ppm					
	Partitioning in µmoles					
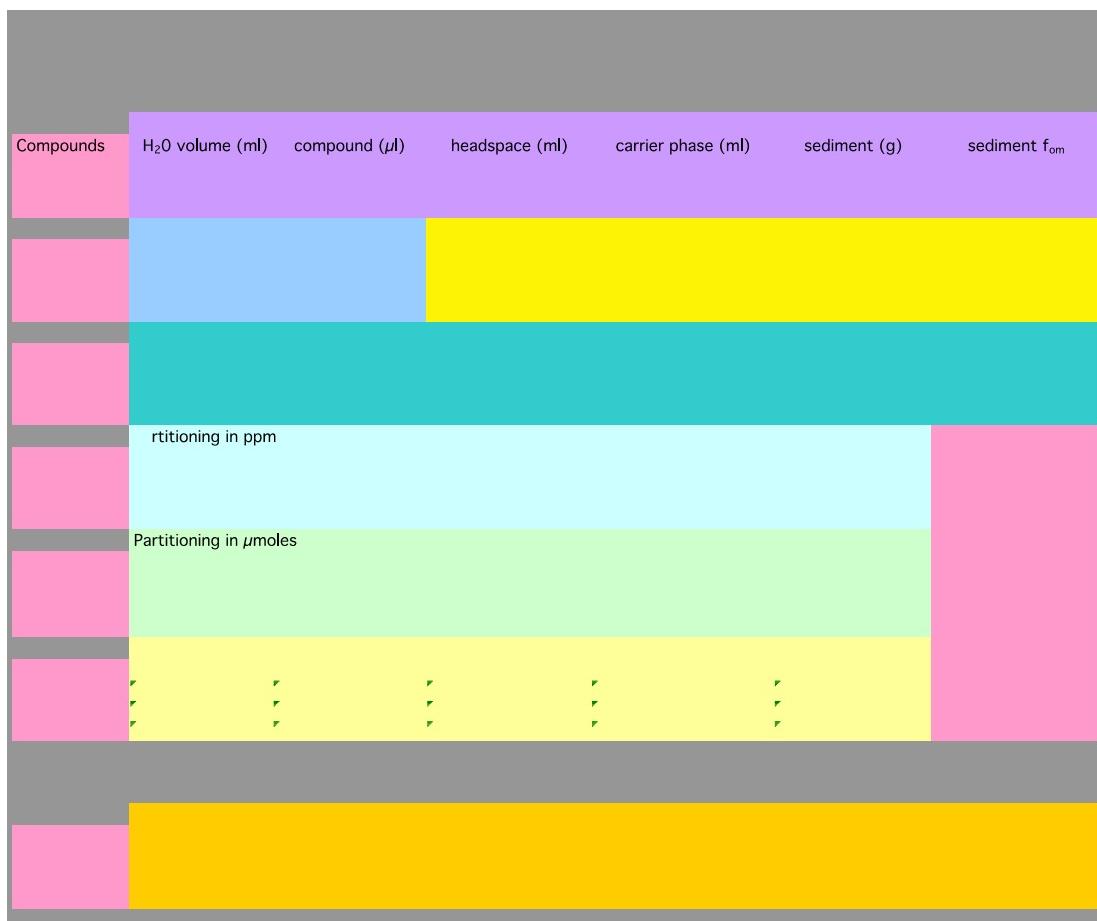
Figure 1.1. Excel spreadsheet for calculating chlorinated solvent partitioning between the aqueous phase, the headspace and NAPL. The figure shows the PCE spreadsheet as an example. Note that NAPL partitioning will only occur when the chlorinated solvent concentration exceeds the aqueous phase solubility.

Assess Substrate Ranges

Knowledge of cultures' substrate ranges was desirable to exploit the high-throughput capabilities of microarray and complementary molecular approaches used in **Task 2 and Task 3** for biomarker gene identification. Furthermore, knowledge of the chlorinated compounds that individual cultures use as electron acceptor(s) for energy conservation allowed detailed studies of substrate interactions (i.e., characterization of 1,1,1-TCA inhibitory effects on the reductive dechlorination of chlorinated ethenes) and also opened the doors for new findings (e.g., the discovery of CF-to-DCM-dechlorinating *Dhb*). Substrate ranges were characterized for available pure cultures and enrichment cultures

containing one or more *Dhc* strains (Table 1.1) (86), the non-methanogenic PCE-to-ethene-dechlorinating consortium Bio-Dechlor INOCULUM (BDI), the methanogenic TCE-to-ethene-dechlorinating consortium KB-1, the 1,1,2,2-TeCA-to-ethene-dechlorinating consortium WBC-2, and the *Dehalobacter*-containing mixed cultures ACT-3 and WL-DCA. Additional efforts explored chlorinated solvent reductive dechlorination in Third Creek enrichment cultures. The Third Creek site is impacted with chlorinated methanes (e.g., CF), chlorinated ethanes (e.g., 1,1,1-TCA), and chlorinated ethenes (e.g., PCE, TCE).

To characterize the range of chlorinated compounds these cultures dechlorinate, the cultures were challenged with different chlorinated methanes, chlorinated ethanes, chlorinated ethenes, chlorinated propanes, chlorinated phenols and chlorinated benzenes. These experiments proved to be challenging due to toxicity effects, the analytical requirements to resolve all possible intermediates, and to distinguish metabolic from co-metabolic dechlorination. Therefore, the scope of this task was reduced and the efforts focused on the following compounds:

- Chlorinated ethenes: Tetrachloroethene (PCE), trichloroethene (TCE), *cis*-1,2-dichloroethene (cDCE), vinyl chloride (VC).
- Chlorinated methanes: Carbon tetrachloride (CT), chloroform (CF), dichloromethane (DCM).
- Chlorinated ethanes: 1,1,2,2-Tetrachloroethane (1,1,2,2-TeCA), 1,1,1-trichloroethane (1,1,1-TCA), 1,1,2-trichloroethane (1,1,2-TCA), 1,2-dichloroethane (1,2-DCA).
- Chlorinated propanes: 1,2-dichloropropane (1,2-DCP).

The results of the substrate range experiments are presented in Table 1.1.

Table 1.1. Substrates dechlorinated by pure cultures and enrichment cultures used in the substrate range experiments. Substrates tested that were not dechlorinated are also indicated. The primary maintenance substrate (S) for each culture is indicated.

Culture or Consortium/ Primary Substrate (S)	Dechlorination Activity (*=Cometabolic)	Chlorinated Substrates TESTED but NOT Used	References
Dhc strain 195 (PCE)	PCE, TCE, cDCE, tDCE* 1,1-DCE → VC*, ethene 1,2-DCA→ ethene 1,2-dibromoethane, 1,2,3,4-tetrachlorodibenzo-dioxin, 2,3,4,5,6-pentachloro-biphenyl, 1,2,3,4-tetrachloro-naphthalene, hexachloro-benzene, 2,3,6-tri-chlorophenol, 1,2,3,4-tetra-chlorodibenzofuran, 2,3-dichlorophenol, 2,3,4-trichlorophenol, poly-brominated diphenyl ethers	Monochlorophenols, 2,3-dichlorodibenzodioxin, 2,3,7,8-tetrachlorodibenzo-dioxin, 2,3,4-trichlorophenol, pentachlorophenol, 2,4-, 2,5-, and 2,6-dichlorophenol	(1, 34, 51, 103, 104, 133)
Dhc strain BAV1 (cDCE)	PCE*, TCE*, cDCE, tDCE, 1,1-DCE → VC, ethene 1,2-DCA, vinyl bromide	Chlorinated propanes, 1,1,1-TCA, 1,1-DCA and CA	(49, 76, 86)
Dhc strain FL2 (TCE)	PCE*, TCE, cDCE, tDCE, → VC*, ethene	1,1-DCE, 1,1,2-TCA, 1,1-DCA, 1,2-DCA, and CA	(53, 86)
Dhc strain GT (TCE)	TCE, cDCE, 1,1-DCE, VC → ethene	PCE, 1,2-DCA, tDCE, CA, 1,1-DCA, 1,1,1-TCA; 1,1,2-TCA, CT, 1,2-DCP; vinyl bromide, 1,1-dichloro-2,2-difluoroethene, 1,2-dichloro-1,2-difluoroethene; 2-chloro-1,1-difluoroethene; 1,1-difluoroethene	(86, 139)
Dehalogenimonas lykanthroporepellens strain BL-DC-9 (1,2,3-TCP)	1,2-DCP → propene 1,2-DCA → VC 1,1,2-TCA → VC 1,2,3-TCP, 1,1,2,2-TeCA	1-CP, 2-CP, 1,2-dichlorobenzene, cDCE, tDCE, PCE, TCE, VC	(86, 161)
Dehalobacter sp. strain RM* enrichment	DCM → acetate	CF, CM	(75)
Geobacter lovleyi SZ (PCE)	PCE → cDCE	cDCE, VC, 1,2-DCA TCA, trifluoroacetic acid, tDCE, 1,1-DCE, CA, 1,1-DCA, 1,2-DCA, 1,1,1-TCA 1,1,2-TCA, 1,2-DCP	(150)

Culture or Consortium/ Primary Substrate (S)	Dechlorination Activity (*=Cometabolic)	Chlorinated Substrates TESTED but NOT Used	References
Geobacter sp. strain KB-1 (PCE)	PCE → cDCE	cDCE, VC, 1,2-DCA	(150)
Culture RC (contains one *Dhc* strain) (1,2-DCP)	1,2-DCP → propene	1,1,2-TCA, 1,2,3-TCP, 1,2-DCA, PCE, TCE, cDCE, tDCE, VC	(85, 86, 126) (unpublished data)
Culture KS (contains one *Dhc* strain) (1,2-DCP)	1,2-DCP → propene	1,1,2-TCA, 1,2,3-TCP, 1,2-DCA, PCE, TCE, cDCE, tDCE, VC	(85, 86, 126) (unpublished data)
OW (contains both *Dhb* and *Dhc*) (PCE)	PCE → ethene 1,2-DCA → ethene	1-CP, 2-CP, 1,2-DCP 1,2,3-TCP, 1,2-DCA 1,1,1-TCA, 1,1,2-TCA, 1,2,3-TCB, 1,2,4-TCB	(24)
Bio-Dechlor INOCULUM (BDI) (Contains *Dhc* strains BAV1, GT and FL2, *Geobacter lovleyi* strain SZ, and a *Dhb* sp. (PCE)	PCE → ethene TCE → ethene cDCE → ethene VC→ ethene 1,2-DCA → ethene 1,1-DCE → ethene	1-CP, 2-CP, 1,2-DCP 1,2,3-TCP, 1,1,1-TCA 1,1,2-TCA, 1,2,3-TCB, 1,2,4-TCB, CT, CF, DCM	(127)
KB-1 TCE (contains a *Geobacter* sp. and at least two *Dhc* strains. (TCE)	PCE → ethene TCE → ethene 1,1-DCE → ethene cDCE → ethene 1,2-DCA→ ethene TCBs → dichlorobenzenes	1,1,1-TCA, 1,1-DCA, 1,1,2-TCA, CT, CF, DCM	(29, 31) (Unpublished data)
KB-1 VC (contains one *Dhc* strain) (VC)	VC → ethene cDCE → ethene TCE → ethene	As for KB-1 TCE + PCE	(30)
ACT-3 (contains two *Dhb* strains) (1,1,1-TCA)	1,1,1-TCA → 1,1-DCA, 1,1-DCA → chloroethane CF → DCM 1,1,2-TCA → VC 1,1,2-trichloro-1,2,2-trifluoroethane → 1,2-dichloro-1,2,2-trifluoroethane	CT, DCM, 1,1,2,2-TeCA, chlorinated ethenes, 1,2-DCA, TCBs, dichlorobenzenes, monochlorobenzene	(43, 44); (154, 155)
1,1-DCA subculture of ACT-3 (contains one *Dhb* strain) (1,1-DCA	1,1-DCA → CA	As for ACT-3 + 1,1,1-TCA; CF	(143)
CF subculture of ACT-3 (contains a *Dhb* strain) (CF)	CF → DCM 1,1,1-TCA → 1,1-DCA	As for ACT-3 +1,1-DCA	(143)

Culture or Consortium/ Primary Substrate (S)	Dechlorination Activity (*=Cometabolic)	Chlorinated Substrates TESTED but NOT Used	References
WL-TCA (contains both *Dhb* and *Dhc* spp.) (1,1,2-TCA)	1,1,2-TCA → ethene 1,1,2,2-TeCA → ethene 1,2-DCA→ ethene PCE → ethene TCE → ethene cDCE → ethene VC→ ethene	1,1,1-TCA, 1,1-DCA, CT, CF, DCM	(47) (Unpublished data)
WL-DCA (contains only *Dhb*) (1,2-DCA)	1,2-DCA→ ethene	As for WL-TCA + 1,1,2-TCA	(46)
WBC-2 contains *Dhb*, *Dhgm* and *Dhc* strains) (1,1,2,2-TeCA, 1,1,2-TCA, cDCE)	PCE → ethene TCE → ethene cDCE → ethene tDCE → ethene 1,1-DCE → ethene VC→ ethene 1,1,2,2-TeCA → ethene 1,1,2-TCA → ethene 1,2-DCA→ ethene	1,1,1-TCA, 1,1-DCA, CT, CF, DCM	(99)
Third Creek enrichments	PCE → ethene TCE → ethene cDCE → ethene VC → ethene 1,2-DCP → propene 1,2-DCA → ethene 1,1,2-TCA → ethene 1,1-DCA → chloroethane 1,1,1-TCA → chloroethane CF → DCM → non-chlorinated products	NA	(135)

NA: Data not available/not confirmed.   *: Cometablic reductive dechlorination

Growth and Maintenance of Dechlorinating Cultures

All cultures were grown with a growth-supporting chlorinated electron acceptor and dechlorination was verified by headspace GC analysis. When >90% of the chlorinated substrates were consumed, the headspace of each culture vessel was flushed with N_2/CO_2 (80/20, vol/vol) to remove volatile organic compounds (VOCs, i.e., residual chlorinated parent compound and daughter products). The absence of VOCs was verified by GC analysis. Individual stock cultures served as inocula for 20 mL vials containing 8 mL of

reduced, bicarbonate-buffered mineral salts medium amended with 5 mM acetate. The vials were sealed with Teflon-lined grey rubber septa and aluminum crimps. Triplicate vials were amended with the same chlorinated electron acceptor from anoxic, sterile, aqueous stock solutions. Two vials received 2 mL inocula from a dechlorinating stock culture and one vial received 2 mL of sterile medium. The final aqueous phase concentration of each chlorinated compound was approximately 0.1 mM in all replicates. Each vial received 3 mL of sterile hydrogen gas and all vials were incubated stationary at room temperature with the stopper down and in the dark. Vials amended with PCE served as positive controls for cultures OW, BDI and KB-1/TCE. Vials amended with VC, 1,1,1-TCA, 1,2-DCA, and 1,2-DCP served as positive controls for cultures KB-1/VC, *Dhb*-TCA, WL, and RC and KS, respectively. GC headspace measurements were obtained 24 hours after inoculation and weekly thereafter.

Dechlorination Activities in Microcosm and Enrichment Cultures

From a bioremediation perspective, the cultures with the broadest substrate range would be considered most utilitarian; however, the enrichment process often selects for the population with the highest dechlorination rates and fastest growth with the chloroorganic substrate(s) available (67). The KB-1 consortium dechlorinated all chlorinated ethenes and 1,2-DCA to ethene and TCBs to dichlorobenzene (see Table 1.1), but no dechlorination was observed when this dechlorinating culture was challenged with 1,1,1-TCA, 1,1-DCA, CF, or 1,2-DCP. Third Creek microcosms yielded enrichment cultures that dechlorinated all chlorinated ethenes to ethene, 1,1,1-TCA to CA, 1,2-DCP to propene, 1,2-DCA and 1,1,2-TCA to ethene and CF to DCM, which was further degraded to non-chlorinated products. Community analysis using high-throughput 454 sequencing of sub-cultures maintained on lower chlorinated substrates (e.g., cDCE, VC) showed that fewer groups of dechlorinating populations are present upon continued enrichment. For example, upon enrichment with chlorinated ethenes, *Dhb* 16S rRNA gene sequences were no longer detected, and *Dhc* 16S rRNA gene sequences were lost upon repeated transfers to medium with 1,1,1-TCA as electron acceptor. The 16S rRNA gene amplicon pyrosequencing data suggested that reductive dechlorination versatility was lost during the enrichment process and populations with specific reductive dechlorination

capabilities were selectively enriched. The ACT-3 culture, which incompletely dechlorinates CF, 1,1,1-TCA, 1,1-2-TCA and 1,1-DCA as well as 1,1,2-trichloro-1,2,2-trifluoroethane, was unable to dechlorinate any chlorinated alkenes and chlorinated aromatic compounds (i.e., chlorinated ethenes and chlorinated benzenes, respectively). This ACT-3 culture contained two closely related *Dhb* strains suggesting co-existence and an overlapping niche among these specialized strains (141, 142). In West Louisiana (WL) cultures, 1,1,2-TCA supported growth of both *Dhb* and *Dhc*, while 1,2-DCA only supported *Dhb,* to the exclusion of *Dhc*. In contrast, the WBC-2 culture contained *Dhb*, *Dhgm* and *Dhc* and dechlorinated chlorinated ethenes, 1,1,2,2-TeCA, 1,1,2-TCA and 1,2-DCA, but despite the presence of *Dhb*, the culture could not utilize 1,1,1-TCA, CF or 1,1-DCA. Furthermore, the *Dhc*-containing consortia KS and RC both dechlorinated 1,2-DCP to propene but were unable to grow with chlorinated ethenes, chlorinated ethanes or other chlorinated propanes as electron acceptors. These consortia contain *Dhc* strain KS and strain RC, respectively, which were implicated in 1,2-DCP-to-propene reductive dechlorination (85, 86, 126). Culture RC was derived from the same sediment that yielded isolate *Dhc* strain FL2, which dechlorinates chlorinated ethenes, but not 1,2-DCP (53). These findings demonstrated that dechlorinators with different substrate specificities co-exist in the same environment, and further suggest that enrichment with different chlorinated electron acceptors not only selects for different genera (e.g., *Dhb*, *Dhc, Dhgm, Geobacter*) and species, but also for different strains of the same species (e.g., *Dhc* strains FL2 and RC). Thus the enrichment process can result in a loss of dechlorinating populations (i.e., lower diversity of dechlorinators) and a culture with reduced substrate range. In general, enrichment cultures maintained with higher chlorinated substrates, or combination of multiple halogenated substrates, have the potential to produce a wider array of dechlorination products and maintain a diversity of dechlorinating microbes with greater metabolic potential.

Implications for Bioaugmentation

Based on these observations, bioaugmentation cultures enriched with chlorinated ethenes are selected for organisms to dechlorinate chlorinated ethenes (e.g., consortium KB-1) and have very likely lost the populations capable of dechlorinating other chlorinated

compounds. The availability of enrichment cultures (e.g., ACT-3, WBC-2, KS, RC, Third Creek) that dechlorinate other chlorinated solvents (e.g., 1,1,1-TCA, CF, 1,2-DCP) offer the opportunity to prepare mixtures ("blends") of cultures with the capability of transforming a wider array of contaminants. Although promising, the robustness of the culture blends needs to be determined on a case-by-case basis.

Identification of Common Co-Contaminants and Possible Interactions

Co-contaminants such as CF and 1,1,1-TCA can affect *Dhc* activity and chlorinated ethene reductive dechlorination. Therefore, biomarkers indicative of co-contaminant degradation and biomarkers that recognize potential inhibitory effects exerted by co-contaminants are of interest. To identify common co-contaminants at sites impacted with chlorinated ethenes, data compiled in existing records and publicly accessible reports were analyzed, and bioremediation consultants and vendors were contacted for their expert opinions. The analysis indicated that the following co-contaminants are frequently encountered at sites where chlorinated ethenes are the primary contaminants (listed in order of detection frequency):

- Chlorinated ethanes (e.g., 1,1,1-TCA)
- Chlorinated methanes (carbon tetrachloride, chloroform, dichloromethane)
- Chlorinated propanes (e.g., 1,2-dichloropropane)
- Chlorobenzenes
- Hexachlorobutadiene
- Chlorofluorocarbons and hydrochlorofluorocarbons
- Petroleum hydrocarbons (heavy oils, BTEX, sometimes MTBE)
- Naphthalene
- 1,4-Dioxane (often in combination with chlorinated ethanes)
- Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and other explosives
- Metals (e.g., chromium, cadmium)
- Metalloids (e.g., arsenic)

The presence, composition and concentration of subsurface contaminants depend on the historic industrial or defense-related activities at each site. A low diversity of contaminants may be encountered at commercial dry cleaner sites or more recently impacted industrial and military sites. Multiple co-contaminants are commonly found at "old" sites that have experienced multiple operational changes over decades of operation and at mixed waste dump sites.

Inhibitory Effect of Co-contaminants on Reductive Dechlorination
Several different combinations of chlorinated compounds were investigated in enrichment cultures to determine the most significant inhibitory effects of common co-contaminants. We first examined the effects of chlorinated ethanes and chlorinated methanes on degradation of chlorinated ethenes (Section A below), and then investigated the effects of chlorinated ethenes on 1,1,1-TCA and CF degradation (Section B below). To this end, the ACT-3 and KB-1 enrichment cultures were investigated alone and in combination with the chlorinated compounds TCE, cDCE, VC, 1,1,1-TCA, 1,1-DCA and CF. Finally, the effects of the chlorinated ethanes 1,1,2-TCA and 1,2-DCA on 1,2-DCP dechlorination were examined in Third Creek microcosms (Section C below).

A. Effect of chlorinated ethanes and chlorinated methanes on the reductive dechlorination of chlorinated ethenes
The effects of the chlorinated ethanes 1,1,1-TCA and 1,1-DCA and the chlorinated methane CF on the dechlorination of chlorinated ethenes had been demonstrated in enrichment cultures (31, 45). CF and 1,1,1-TCA were inhibitory to reductive dechlorination of chlorinated ethenes, while 1,1-DCA was a less potent inhibitor. Thus blending a 1,1,1-TCA-dechlorinating culture (e.g., ACT-3) with a *Dhc*-containing chlorinated ethene-dechlorinating culture (e.g., KB-1) resulted in removal of the inhibition and complete dechlorination of chlorinated ethenes to ethene was achieved (45). The success of this culture combination prompted further investigations into possibilities to relieve inhibition by blending cultures with complementary dechlorinating abilities.

CF inhibits many microbial processes, including methanogenesis and reductive dechlorination (31, 45). A survey of laboratory cultures explored whether some cultures were more resistant to inhibition by CF. While testing the 1,1,1-TCA-to-CA-dechlorinating culture ACT-3, we discovered that not only was this culture resilient to CF, it was also capable of dechlorinating CF to DCM. The demonstration of organohalide respiration with CF by a *Dhb* population in the ACT-3 culture is presented below.

CF was shown to inhibit the dechlorination of chlorinated ethenes in culture KB-1 at concentrations lower than 6.7 μM (31). The objective of this experiment was to evaluate whether a CF-exposed KB-1 culture could recover dechlorination activity after CF removal. Following exposure to high CF concentrations (230-250 μM) for 15-20 days (during which none of the chlorinated ethenes were dechlorinated), KB-1 cultures were purged with H_2/CO_2 (80/20, vol/vol) for 2 hours to remove all CF and chlorinated ethenes. The purged KB-1 cultures were amended with TCE, cDCE or VC (Figure 1.2). Cultures not exposed to CF dechlorinated TCE in 5-6 days. Exposure to high concentrations of CF for 15 days resulted in significantly slower dechlorination, particularly for cDCE and VC (Figure 1.2B and 1.2C)

B. *Effect of chlorinated ethenes on chlorinated ethane and CF dechlorination*
To investigate if chlorinated ethenes inhibited chlorinated ethane or methane dechlorination, culture ACT-3 was amended with various concentrations of TCE, cDCE and VC. VC was found to be the strongest inhibitor of CF dechlorination in culture ACT-3. CF dechlorination to DCM was not inhibited with 35 μM TCE, while 265 μM TCE completely inhibited dechlorination. CF dechlorination was completely inhibited by cDCE at both high (265 μM) and low (35 μM) concentrations, indicating that cDCE had a greater inhibitory effect than TCE. Most pronounced was the impact of VC on CF dechlorination. Even at VC concentrations as low as 0.96 μM, a decrease in the CF dechlorination rate was observed (Figure 1.3).
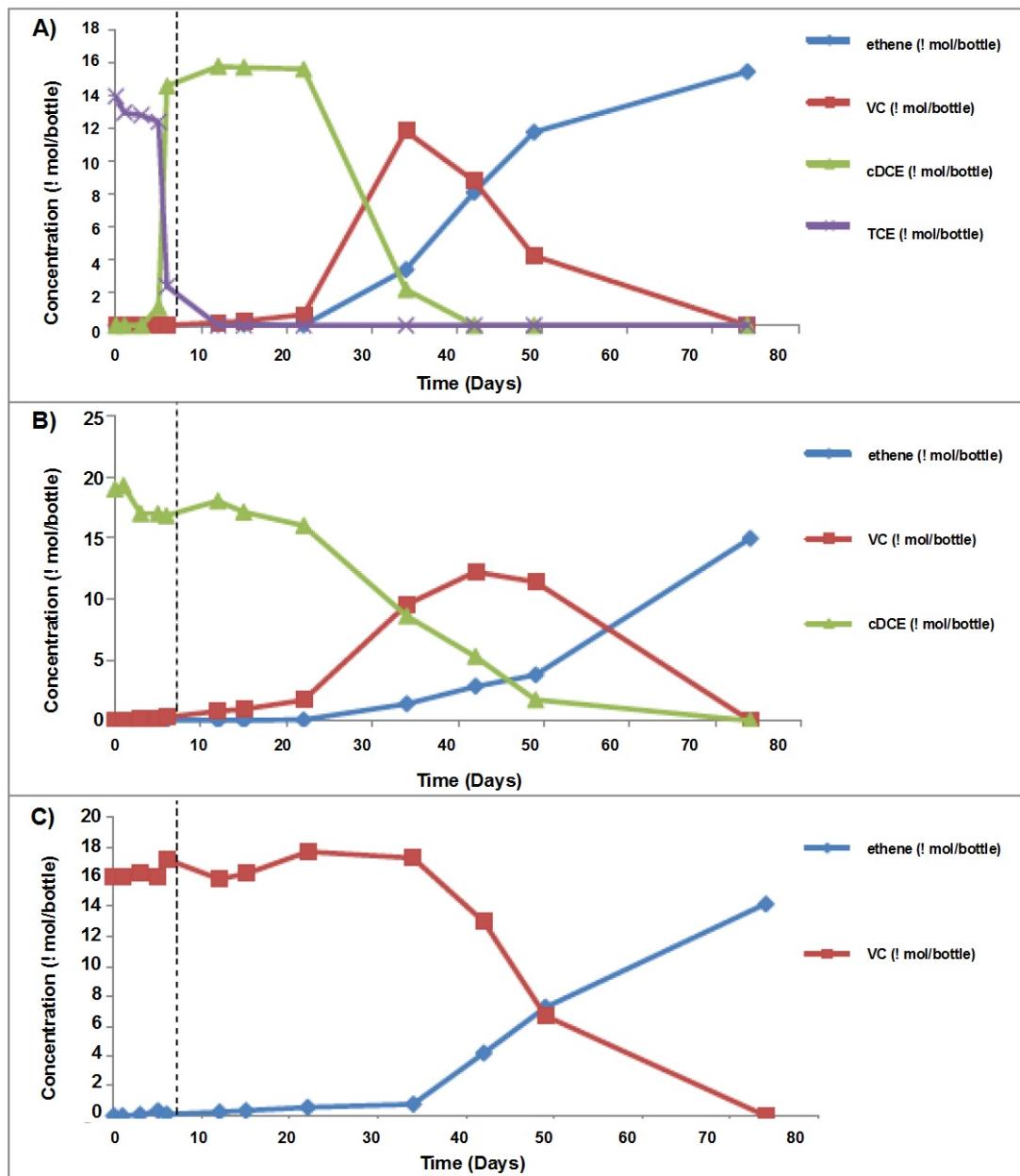
Figure 1.2.   TCE, cDCE and VC dechlorination in KB-1 after exposure to CF.
A) KB-1 culture amended with TCE after removing CF. B) KB-1 culture amended with cDCE after removing CF. C) KB-1 culture amended with VC after removing CF. The dashed lines indicate when positive controls completely dechlorinated the corresponding chlorinated ethene to ethene.
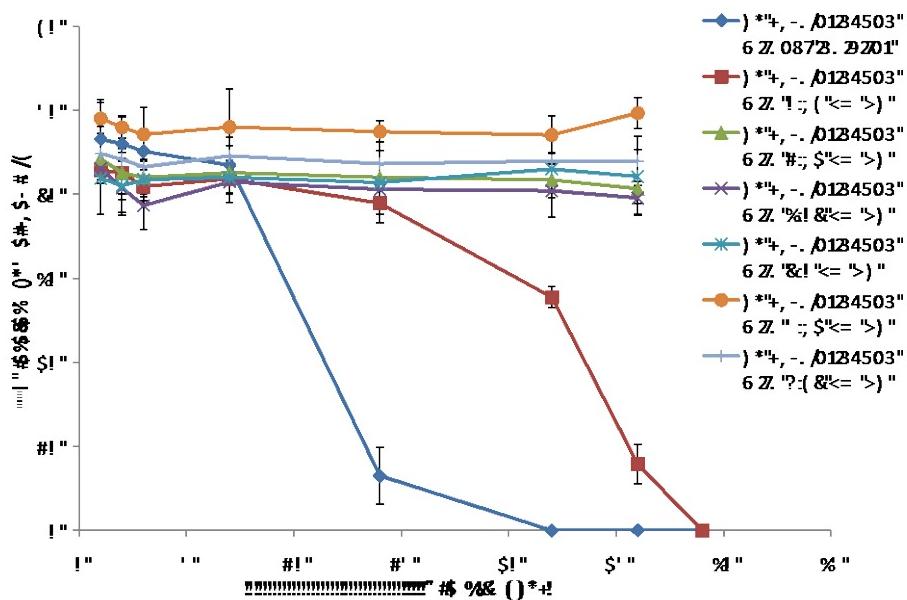
Figure 1.3. CF Dechlorination in ACT-3 cultures in the presence of increasing concentrations of VC.
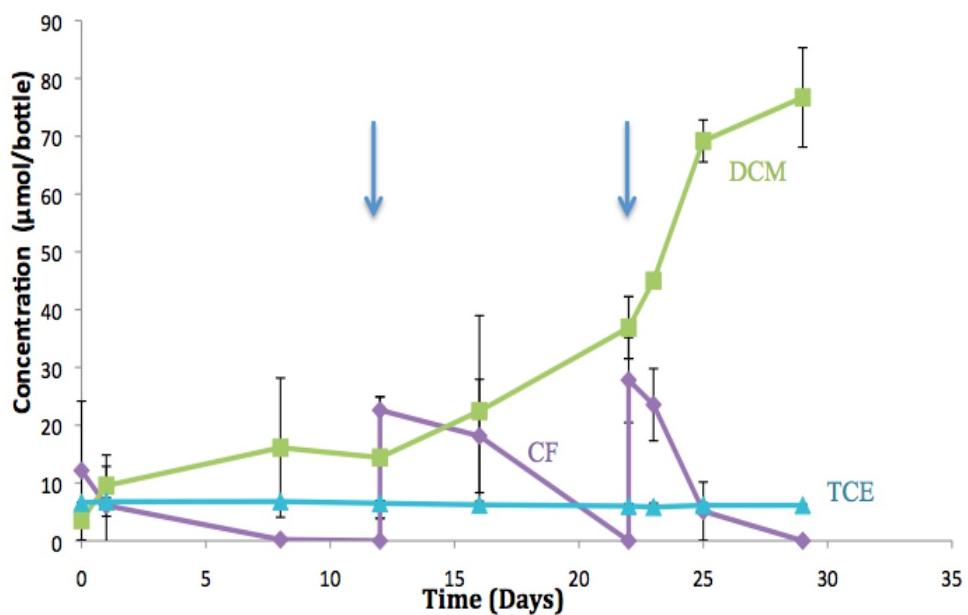
Figure 1.4. CF dechlorination to DCM in the ACT-3 culture in the presence of 30 μM TCE. The arrows indicate when the ACT-3 culture was amended with additional CF and electron donor.

Since VC dechlorination to ethene was most strongly inhibited by CF in KB-1 cultures (Section A above), and conversely, CF-to-DCM dechlorination was inhibited by VC in ACT-3 cultures, the dechlorination performance of culture blends was tested. When the cultures were tested individually, CF dechlorination to DCM in ACT-3 cultures was not affected by 30 µM TCE (Figure 1.4); however, VC dechlorination in KB-1 cultures was significantly inhibited by 30 µM CF (Figure 1.5).
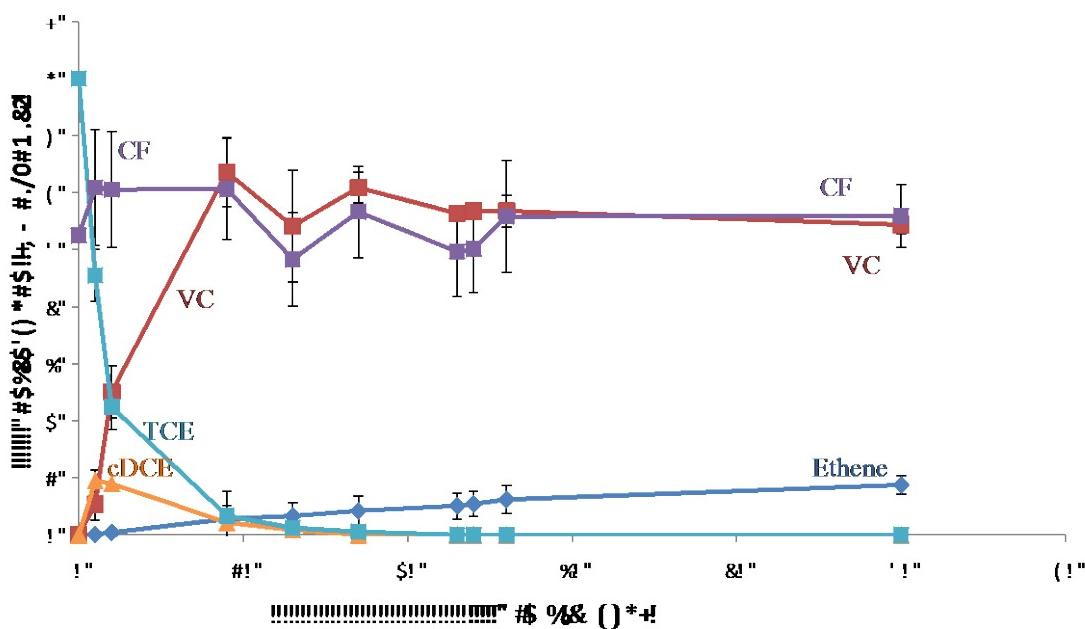
Figure 1.5. TCE dechlorination in culture KB-1 in the presence of 30 µM CF.

When a 50:50 (vol/vol) ACT-3 and KB-1 culture blend was amended with TCE and CF (30 µM each), 3 µmol/bottle CF were dechlorinated to DCM after one day of incubation, but further dechlorination of CF did not occur over a 2-month incubation period. TCE and cDCE were dechlorinated to VC, yet further dechlorination to ethene was extremely slow (Figure 1.6). Neither CF dechlorination nor TCE dechlorination went to completion because the VC produced from TCE dechlorination inhibited CF dechlorination before CF concentrations were reduced sufficiently to no longer impact VC dechlorination. Hence, a "stalemate" condition was reached, with both VC and CF remaining. A possible strategy to overcome this "stall" could be the continued inoculation (multiple

times) with a CF-dechlorinating culture to reduce the CF concentrations below inhibitory levels, since with each inoculation, a fraction of CF is dechlorinated before the culture becomes inhibited.
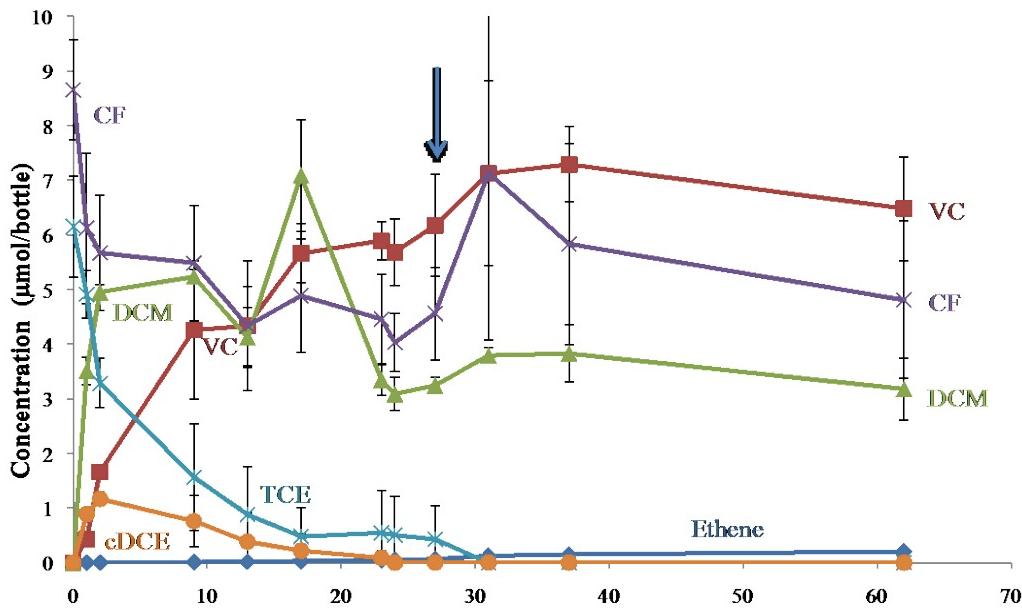
Figure 1.6. Simultaneous dechlorination of CF and TCE in a blend of cultures KB-1 and ACT-3. Dechlorination of CF to DCM only occurred before VC accumulated in the first 1-2 days. VC dechlorination was inhibited by the remaining CF resulting in incomplete dechlorination.

C. Co-contaminant effects in Third Creek microcosms

Dechlorinating enrichment cultures were obtained from microcosms established with chlorinated solvent-impacted Third Creek sediment collected in Knoxville, TN. Enrichment with chlorinated ethenes, CF, and 1, 2-dichloropropane (1,2-DCP) yielded cultures with different substrate utilization patterns. To explore the co-contaminant effect on 1,2-DCP dechlorination in Third Creek enrichments, three sets of cultures were initiated: a) cultures amended with 0.5 mM 1,2-D, b) cultures amended with 0.5 mM 1,2-DCP and 0.5 mM 1,1,2-TCA, and c) cultures amended with 0.5 mM 1,2-DCP and 0.5 mM 1,2-DCA. All cultures received 5 mM lactate as electron donor. Cultures that received 1,2-DCP only produced stoichiometric amounts of propene after 1 month of

incubation. Sequential dechlorination was observed in the microcosms amended with 1,2-DCP and a chlorinated ethane, where chlorinated ethanes were dechlorinated first, followed by 1,2-DCP reductive dechlorination to propene. In cultures amended with 1,1,2-TCA, dichloroelimination to VC occurred, which was further dechlorinated to ethene. The presence of VC had no inhibitory effect on 1,2-DCP dechlorination to propene.

In summary, co-contaminant experiments are important to predict contaminant fate and the potential success of bioremediation approaches at sites impacted with more than one chlorinated solvent. Considering that most sites impacted by chlorinated ethenes have mixed contaminant plumes, further investigations of the effects of common co-contaminants on *Dhc* and other keystone dechlorinators are warranted.

Discovery of a tDCE-Dechlorinating *Dehalogenimonas* Strain

The WBC-2 enrichment culture was initially developed at the USGS from West Branch Canal Creek-derived microcosms maintained with TeCA, 1,1,2-trichloroethane, and cDCE as electron acceptors (74). Culture WBC-2 dechlorinates 1,1,2,2-tetrachloroethane (TeCA) to ethene. The dominant pathway in culture WBC-2 followed a dihaloelimination reaction yielding tDCE, which was subsequently transformed to VC and then to ethene via reductive dechlorination (i.e., hydrogenolysis) (99) (Figure 1.7).

Analysis of 16S rRNA gene clone libraries from culture WBC-2 DNA revealed sequences from three putative dechlorinating organisms belonging to the *Dhc*, *Dhb*, and *Dhgm* genera. Sub-enrichment cultures with each of the putative chlorinated intermediates (i.e., daughter products) were established, and the abundance of each dechlorinating genus was determined in these sub-enrichments using genus-specific qPCR assays (see Task 3, Table 3.1). *Dhgm* was found only in cultures amended with TeCA or tDCE (Figure 1.8) (99).
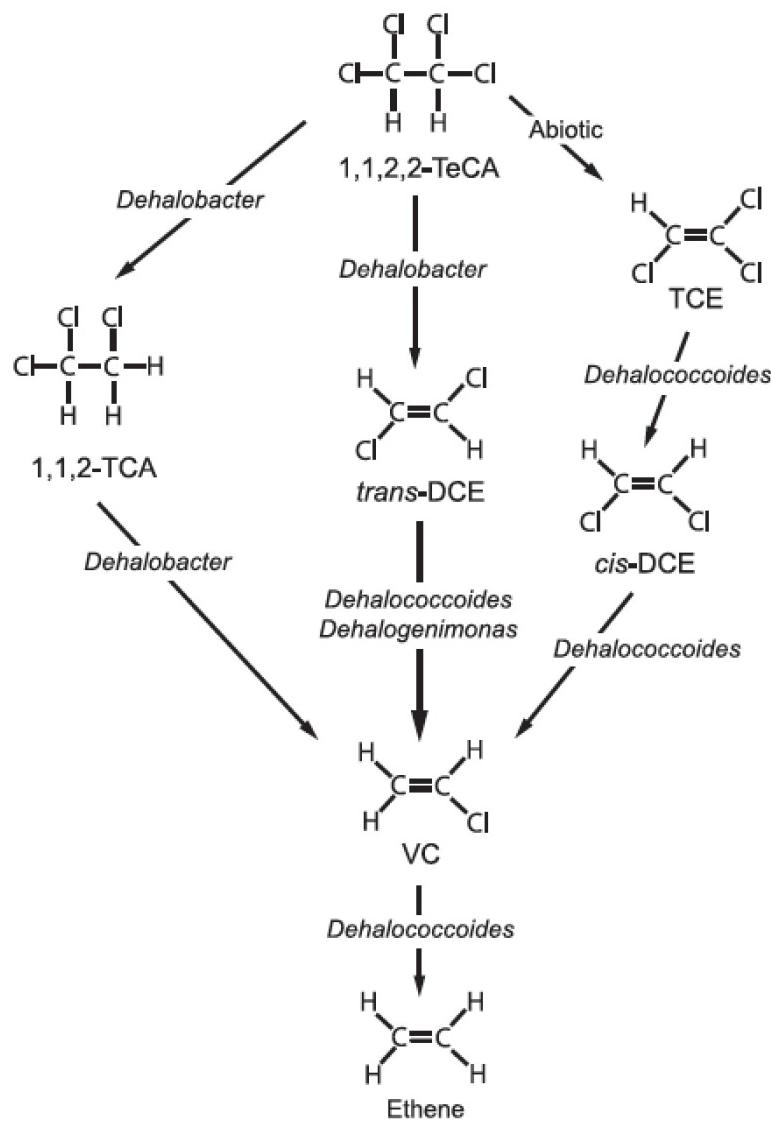
Figure 1.7. Summary of 1,1,2,2-TeCA dechlorination pathways and organisms in the WBC-2 culture. 1,1,2,2-TeCA is primarily dechlorinated to tDCE. Alternative pathways leading to the production of TCE or 1,1,2-TCA were observed when the WBC-2 culture has been perturbed (i.e., oxygen exposure, dilutions).
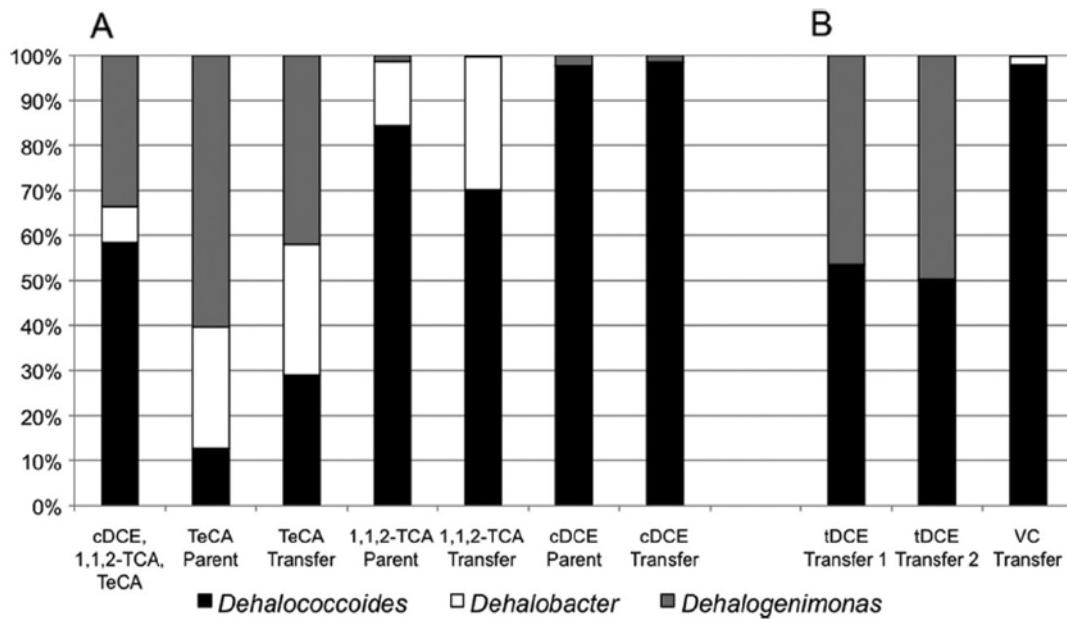
FIG 1.8. Relative abundance of dechlorinating genera in the WBC-2 sub-cultures maintained with different substrates. Relative abundance was calculated from qPCR data with genus-specific primer sets. One hundred percent represents approximately 2×10^7 16S rRNA gene copies/mL of culture. (A) WBC-2 cultures enriched with a specific chlorinated compound as electron acceptor over the course of 18 months. (B) Transfer cultures enriched with tDCE or VC as electron acceptor over the course of an 8-month incubation period. Each enrichment culture had dechlorinated a minimum of 16 amendments of chlorinated acceptor prior to DNA extraction and qPCR (99).

An increase in the abundance of each genus was observed in the enrichment cultures during incubation (Figure 1.9). These data revealed that complete dechlorination of TeCA to ethene involved all three organisms. *Dhb* 16S rRNA genes increased during the dihaloelimination of TeCA to tDCE, while *Dhc* and *Dhgm* 16S rRNA genes increased during hydrogenolysis of tDCE to VC, and *Dhc* 16S rRNA genes increased during hydrogenolysis of VC to ethene. This is the first time a genus other than *Dhc* has been implicated in dechlorination of tDCE to VC (99).

The *Dhgm* strain in the WBC-2 culture was assigned to the phylum *Chloroflexi* and shared 91% 16S rRNA gene sequence identity with *Dhc* and 96% sequence identity with the recently described *Dhgm* strains BL-DC-9 and BL-DC-8 (108). This level of sequence similarity places the WBC-2 16S rRNA gene sequence within the *Dhgm* genus, but perhaps as a distinct species from the published *Dhgm lykanthroporepellens* and

Dhgm alkenigignens. These characterized *Dhgm* species dechlorinate chlorinated propanes and chlorinated ethanes, but cannot dechlorinate chlorinated ethenes (15, 108).
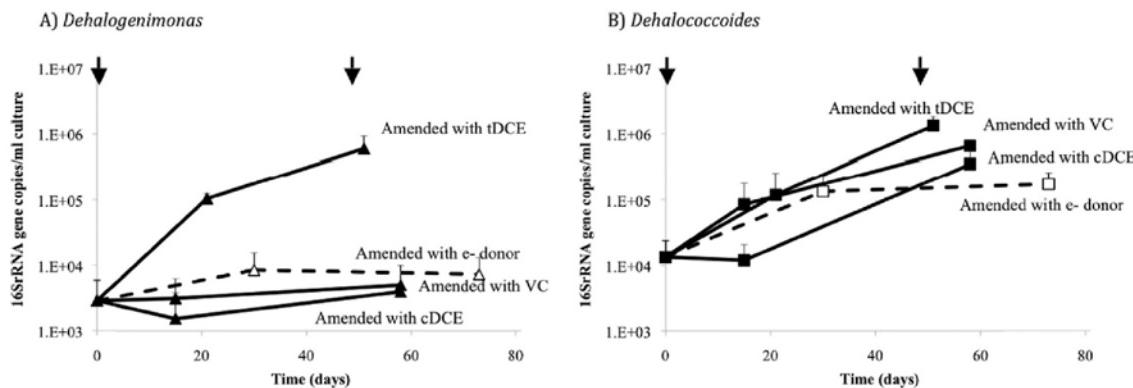
FIG 1.9. Growth of *Dhc* and *Dhgm* during dechlorination of cDCE, tDCE, and VC in 1:20 diluted culture. (A) *Dhgm* 16S rRNA gene copies/mL and (B) *Dhc* 16S rRNA gene copies/mL in separate cultures amended with tDCE, cDCE, VC, or electron donor only. Closed symbols indicate the results for cultures amended with both electron donor and acceptor, and open symbols with dashed lines indicate results for controls amended with the electron donor only (99).

Growth-Linked Dechlorination of CF to DCM by *Dehalobacter*

In an experiment designed to quantify the anticipated inhibitory effects of CF on 1,1,1-TCA dechlorination in mixed culture ACT-3 (also referred to as culture *Dhb*-TCA), transformation of CF to DCM was observed. When CF was added to triplicate subsamples of this culture never previously exposed to CF, reductive dechlorination of CF began immediately (45). Up to 500 µM (~60 mg/L) of CF were stoichiometrically converted to DCM in 29 days (Figure 1.9). Dechlorination rates increased with subsequent additions of CF while at the same time methanogenesis diminished (Figure 1.10). CF dechlorination has been sustained in cultures maintained with CF and either a methanol, ethanol, and lactate (MEL) mixture or H_2 as electron donors for several years, including a series of sequential 2% transfers into fresh medium. The highest CF dechlorination rate observed was 360 µM/day in a culture maintained with 1 mM CF. The predominant phylotypes in the *Dhb*-TCA culture were quantified by qPCR over several CF or 1,1,1-TCA amendments. These experiments confirmed that *Dhb* grew

during CF dechlorination to DCM at similar yields as it grew with 1,1,1-TCA. Sustained

dechlorination required purging with N_2/CO_2 (80/20, vol/vol) once DCM concentrations

exceeded 2 mM, suggesting that DCM above 2 mM was inhibitory to the CF

dechlorinator (44). Of note, none of the cultures tested (nor any in the published

literature) were capable of dechlorinating CT in a growth-linked fashion. In fact, CT was

a potent inhibitor oc CF reductive dechlorination and no DCM was fomed in ACT-3

cultures amended with small amounts (2.5 mg/L) of CT.

Figure 1.10. Dechlorination of CF to DCM by a *Dhb* population in mixed culture ACT-3 (*Dhb*-TCA). CF, closed circles; DCM, open circles; methane, open triangles and dashed lines. The culture was amended with 0.5 mM (60 mg/L) CF. Points show the average of triplicate cultures with error bars showing one standard deviation. Asterisks denote when cultures were purged with N_2/CO_2 and amended with additional CF, acetate and the MEL electron donor mixture (44).

CF and 1,1,1-TCA share the trichloromethyl group and we investigated whether the same

enzyme system catalyzed the reductive dechlorination of both compounds. Using an

established enzyme assay protocol (46), RDase activity was assayed in cell-free extracts

prepared from *Dhb*-TCA and subcultures maintained with CF (*Dhb*-CF) and DCA (*Dhb*-

DCA). Michaelis-Menten kinetic parameters for each substrate and culture combination

were determined (Figure 1.11).
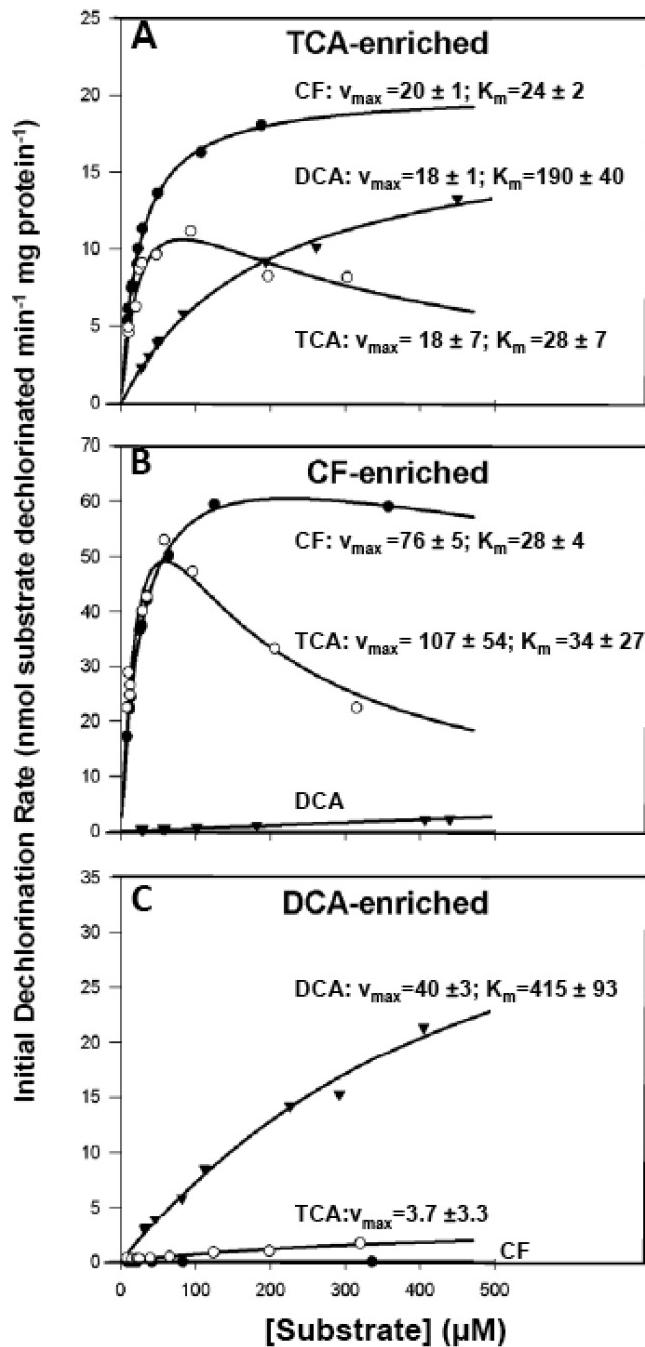
Figure 1.11. Kinetics of reductive dechlorination in cell-free extracts prepared from the parent culture *Dhb*-TCA (A) and subcultures *Dhb*-CF (B) and *Dhb*-DCA (C), enriched on the growth substrates TCA, CF, and DCA, respectively. Points represent initial dechlorination rates determined from individual sacrificial dechlorination assay vials with 1,1,1-TCA (open circles), CF (closed circles) or 1,1-DCA (closed triangles) as assay substrates. Lines represent best fits of the data to the Michaelis-Menten model (incorporating the Haldane model for substrate inhibition for 1,1,1-TCA and CF at high initial substrate concentrations as described previously (23). Corresponding kinetic parameters are V_{max} (nmol substrate dechlorinated min^{-1} mg $protein^{-1}$) and K_m (µM) (± 95% confidence intervals).

The kinetic data were consistent with CF and 1,1,1-TCA being dechlorinated by the same enzyme system (44). The two sub-cultures developed from the ACT-3/*Dhb*-TCA (1,1,1-TCA-fed) parent culture (*Dhb*-CF and *Dhb*-DCA) were fed CF and 1,1-DCA, respectively, and developed more restricted substrate ranges, consistent with the observed dechlorination activity in the corresponding cell-free extracts. The parent culture *Dhb*-TCA dechlorinated 1,1,1-TCA, CF, and 1,1-DCA, while *Dhb*-DCA only dechlorinated DCA, but not 1,1,1-TCA or CF. *Dhb*-CF dechlorinated 1,1,1-TCA to DCA and CF to DCM at similar rates, but did not further dechlorinate DCA to CA. Additional halogenated substrates were tested. The *Dhb*-TCA culture did not dechlorinate PCE, TCE, cDCE, VC, 1,1,2-TCA, or 1,2-DCA. Some CT was dechlorinated abiotically to CF in autoclaved and reduced culture medium and inoculation with the CF-dechlorinating culture did not increase CT dechlorination. Further investigations of this culture using pyrotag and metagenome sequencing (143), as well as Blue Native PAGE gel electrophoresis (142), corroborated that the *Dhb* populations in the CF- and 1,1,1-TCA-dechlorinating cultures possess the same RDase. In particular, two distinct but closely related *Dhb* strains are present in the parent culture, one that dechlorinates 1,1,1-TCA and CF, and the other 1,1-DCA to CA (Figure 1.12). These data are consistent with the existence of two different RDases catalyzing these two reactions, as was postulated previously (44). These RDases are further described in Task 2.
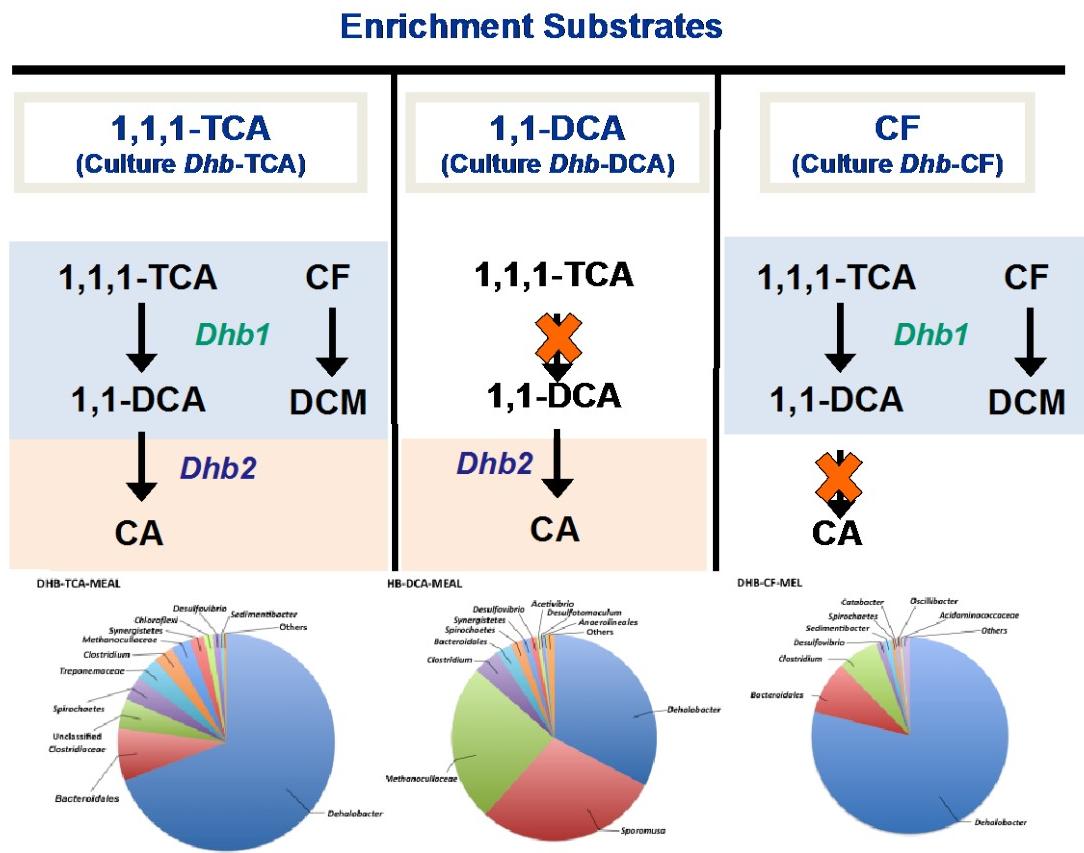
Figure 1.12. Identification of two *Dhb* strains (Dhb1 and Dhb2) in culture ACT-3 (*Dhb*-TCA). Metagenome sequencing and biochemical analysis of the parent culture (enriched with 1,1,1-TCA) and the two sub-cultures (enriched with 1,1-DCA or CF) established the presence of two *Dhb* strains. Pie charts present results of pyrotag sequencing of the 16S rRNA genes in each of the parent and both sub-cultures. All three cultures were dominated by *Dhb* (blue), but the 1,1-DCA enrichment also contained methanogens (green) and an acetogen (*Sporomusa*; red).

Growth-Linked DCM Degradation by a Unique *Dehalobacter* Population

Culture *Dhb*-TCA (ACT-3) reductively dechlorinates CF to DCM, which is not transformed further. DCM can be degraded under oxic conditions but CF-to-DCM reductive dechlorination is a strictly anaerobic process indicating that DCM is formed in anoxic plumes. To explore the fate of DCM in the absence of oxygen, pristine freshwater sediment from Rio Mameyes in Luquillo, Puerto Rico, was used to establish microcosms inside an anoxic chamber filled with H_2/N_2 (3%/97%, vol/vol) as described (52). Each 12-mL aliquot of sediment slurry was dispensed into sterile 24-mL vials, and received 20

mg L^{-1} DCM (~128 µM aqueous concentration). Triplicate microcosms were incubated statically at room temperature in the dark. Standard curves were prepared by adding known amounts of DCM (1.5 µM to 385 µM) or CM (85 µM to 3,415 µM) to culture vessels containing sterile medium. DCM and CM were analyzed using a GC equipped with flame ionization and electron capture detectors. Methane, acetate, and formate were monitored as described (7). *Dhb* 16S rRNA genes were quantified with qPCR as described (47).

DCM was consumed in live microcosms and additional doses of DCM were consumed at increasing rates. Repeated transfers to fresh medium yielded sediment-free enrichment cultures. DCM in the 10th transfer culture was consumed at a rate of 4.0 mg L^{-1} day^{-1} and cultures tolerated up to 200 mg L^{-1} DCM without apparent inhibition. CT and CF inhibited both DCM utilization and methanogenesis, while the addition of 2-bromoethane sulfonate (BES) inhibited only methane production without preventing DCM degradation (75). No CM formation was observed during DCM degradation and CM was not degraded when supplied as a substrate. The DCM-fed cultures produced acetate suggesting that DCM was fermented. 16S rRNA gene amplicon sequencing demonstrated the increase in *Dhb* sequences and qPCR targeting the *Dhb* 16S rRNA gene corroborated that a *Dhb* population gew at the expense of DCM fermentation. *Dhb* 16S rRNA gene copy numbers increased to $> 2 \times 10^7$ mL^{-1} as DCM was consumed, yielding $2.9 \pm 1.1 \times 10^5$ *Dhb* 16S rRNA gene copies per µmol of DCM consumed. When DCM feedings were stopped, the *Dhb* 16S rRNA gene copy numbers decreased to less than 10^6 cells mL^{-1} (Figure 1.13) and increased again to $> 10^7$ cells mL^{-1} when additional DCM was provided (not shown). Attempts to grow the DCM-fermenting *Dhb* sp. via CO_2/H_2 reductive acetogensis failed suggesting the DCM degrader is not a homoacetogen (75). DNA from a DCM-degrading enrichment culture was used to establish a 16S rRNA gene-based clone library and 208 clones were screened. Of these clones, 105 carried an ~1,500 bp long 16S rRNA gene fragment, 81 of which were identified as *Dhb* sequences. The nearly full-length (1,480 bp) sequences of six randomly chosen *Dhb*-positive clones shared 97% identity with clone sequence CK10 (1333/1381 positions; GU320656) and 95% identity with *Dhb* sp. 1,1-DCA1 (1352/1421 positions; DQ777749), as illustrated in the phylogenetic tree shown in Figure 1.14.
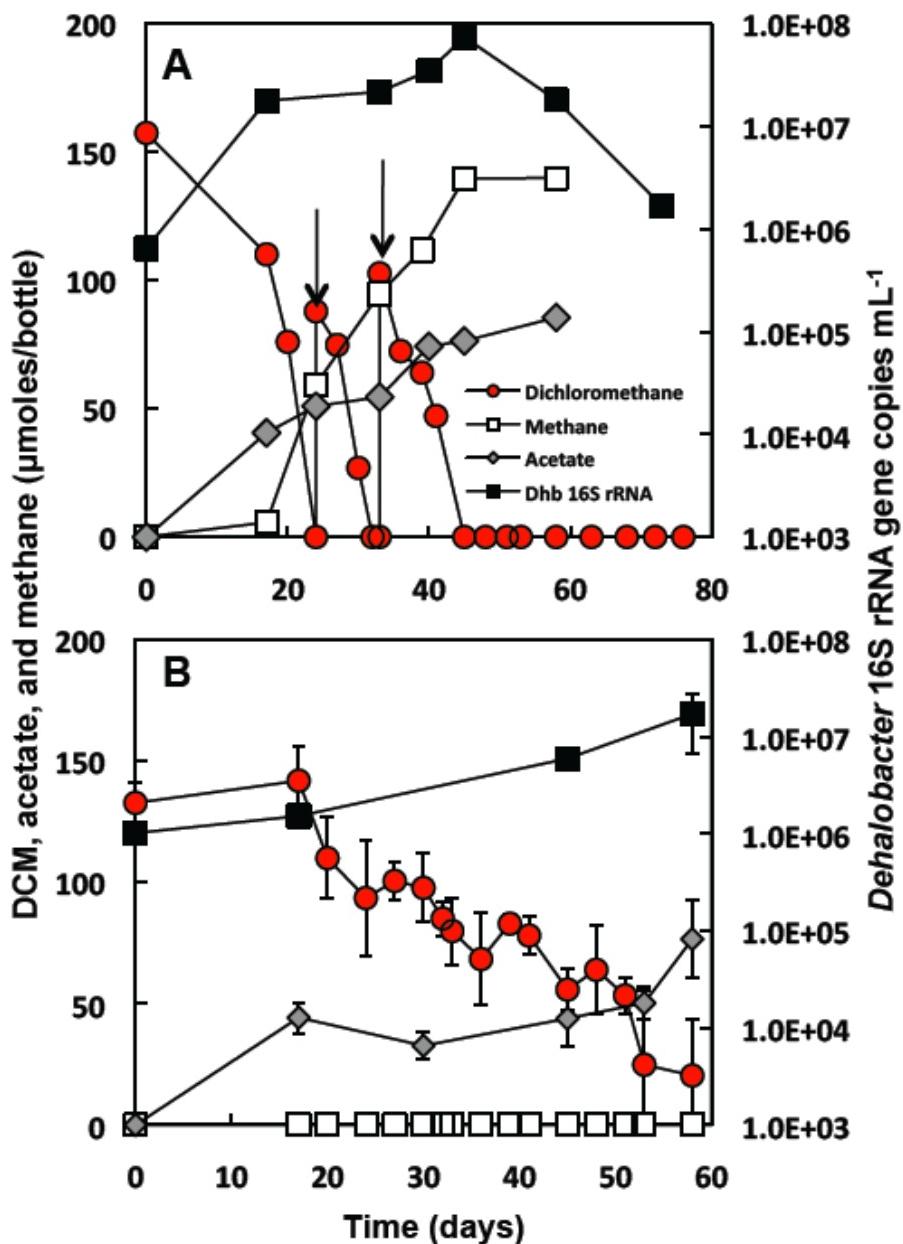
Figure 1.13. Degradation of DCM in the Rio Mameyes enrichment culture: (A) *Dehalobacter* sp. 16S rRNA gene copies per mL increases as DCM is degraded and decrease once DCM is completely gone. (B) BES-amended incubations demonstrate that methanogens are not involved in DCM degradation. Note: No *Dhb* growth occurred in controls inoculated with the DCM-degrading enrichment but without DCM addition.
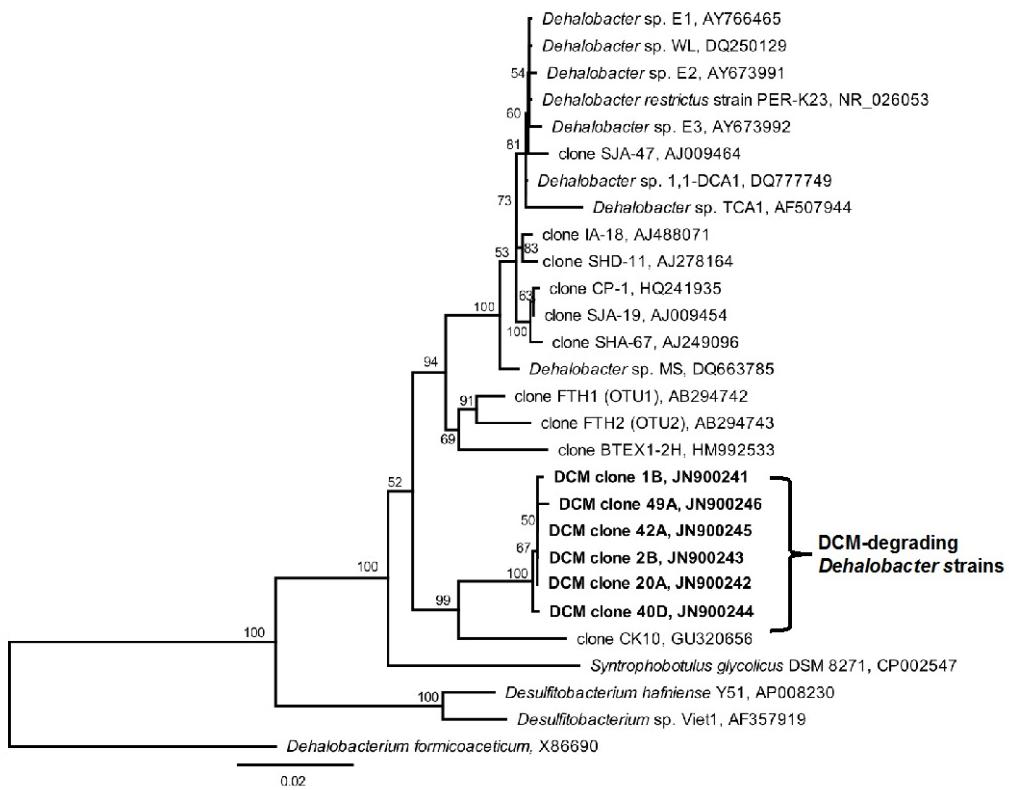
Figure 1.14. 16S rRNA gene-based phylogenetic illustrating the affiliation of the DCM-fermenting organism with the *Dhb* genus. Included in the analysis were nearly full-length (1,480 bp) sequences from six randomly chosen *Dhb*-positive clones. These sequences shared 97% identity with clone sequence CK10 (1333/1381 positions; GU320656) and 95% identity with *Dhb* sp. strain 1,1-DCA1 (1352/1421 positions; DQ777749).

The new *Dhb* 16S rRNA gene sequences were submitted to GenBank under accession numbers JN900241-246. The predominance of *Dhb* 16S rRNA gene fragments in the clone library suggested that one or more *Dhb* strains were involved in DCM degradation. Of the 24 clones with non-*Dhb* 16S rRNA gene inserts, about half of the fragments were *Acetobacterium* sp. sequences. The remaining clones were most similar to environmental clone sequences without cultured representatives (75). The DCM-degrading culture failed to reductively dechlorinate CF, suggesting that different *Dhb* populations are responsible for CF reductive dechlorination and DCM fermentation. The finding of a DCM-fermenting *Dhb* strain extends the metabolic range of the *Dhb* genus, which so far has been restricted to organohalide respiration. We propose the term "organohalide fermentation" to describe the metabolic process of DCM degradation under anoxic conditions.

Elucidating Substrate Interactions at the Cell and Enzyme Level

The goal of this task was to explore the nature of the inhibitory effects caused by 1,1,1-TCA, 1,1-DCA, and CF on *Dhc* activity in more detail. Further, the effects of chlorinated ethenes on 1,1,1-TCA and CF transformation were explored as these are among the most common co-contaminants at chlorinated ethene-contaminated sites. The goal was to determine if the observed inhibition in growing cultures was related to interactions specifically with RDases (i.e., at the enzyme level), or if the inhibition resulted from interactions with other cellular components (i.e., at the cellular level). To explore inhibitory effects at the cellular and enzyme levels, whole cell suspensions (resting cell suspensions) using hydrogen as the electron donor, as well as cell-free extract dechlorination assays (with reduced methyl viologen as artificial electron donor) were performed. In all, four different experiments were conducted using whole cell suspensions and cell-free extracts to investigate inhibition kinetics. Specifically, experiments were conducted to explore the:

1. Effect of 1,1,1-TCA and 1,1-DCA on TCE, cDCE and VC dechlorination in three mixed cultures (OW, BDI and KB-1);
2. Effect of chlorinated ethenes (TCE, cDCE and VC) on 1,1,1-TCA and 1,1-DCA dechlorination in the ACT-3 culture;
3. Effect of chlorinated ethenes (TCE, cDCE and VC) on CF dechlorination in the ACT-3 culture; and
4. Effect of CF on TCE, cDCE and VC dechlorination in the KB-1 culture.

All dechlorination assays were performed in an anoxic chamber in 2 mL glass vials with Teflon-lined caps. Cultures were prepared for whole cell suspension assays by purging each culture free of chlorinated compounds with N_2/CO_2 (80:20, vol/vol) for 15 to 20 minutes. Culture suspensions (75 µL) were aliquoted into mineral salts medium supplemented with 5 mM acetate and purged with H_2/CO_2 (80:20; hydrogen is the electron donor) to a final total volume of about 2 mL. To prepare cell-free extracts, culture suspensions were centrifuged, the cell pellets suspended in buffer, and the cells lysed by sonication. Resulting crude cell-free extracts (10 to 30 µL) were added to assay buffer containing 100 mM Tris-HCl, pH 7.4, 2 mM methyl viologen (artificial electron

donor), and 2 mM titanium (III) citrate to a final volume of about 2 mL. Chlorinated compounds were added to the cell suspensions and cell-free extract assay vials from neat or aqueous stock solutions to achieve the targeted concentration ranges. Assays were allowed to incubate for 1-3 hours, during which less than 10% of parent compound was dechlorinated, and the accumulation of dechlorination products was quantified by GC/FID. This strategy provided an accurate determination of initial dechlorination rates without a significant change in initial chlorinated substrate concentrations. The initial dechlorination rate (nmol substrate dechlorinated/min/vial) for each permutation was determined and then normalized to the total protein in each vial to obtain the specific initial dechlorination rate (v_o) in units of nmol substrate dechlorinated/min/mg protein. Michaelis-Menten kinetic parameters, including half saturation constant (K_m), maximum specific dechlorination rate (V_{max}) and inhibition constant (K_i) were determined with a nonlinear regression model for each culture/substrate/inhibitor combination. The Enzyme Kinetics 1.3 Module from SigmaPlot 10 (Systat Software Inc., Chicago, IL) was used for analysis. Each data set for each culture/substrate/inhibitor combination was tested using the competitive, uncompetitive and noncompetitive inhibition equations as described below:

- Basic Michaelis-Menten equation: $v_o = \dfrac{V_{max}[S]}{K_m+[S]}$

- Competitive inhibition model: $v_o = \dfrac{V_{max}}{1+\frac{K_m}{[S]}(1+\frac{[I]}{K_{ic}})}$

  - only Km affected in the competitive inhibition model: $K_m^{app} = K_m(1+\frac{[I]}{K_{ic}})$

- Uncompetitive inhibition model: $v_o = \dfrac{V_{max}}{1+\frac{[I]}{K_{iu}}+\frac{K_m}{[S]}}$

  - Km and Vmax both affected in the uncompetitive model:

  $K_m^{app} = \dfrac{K_m}{1+\frac{[I]}{K_{iu}}}$ and $v_{max}^{app} = \dfrac{V_{max}}{1+\frac{[I]}{K_{iu}}}$

- Non-competitive inhibition model: $v_o = \dfrac{V_{max}}{\left(1+\frac{[I]}{K_{in}}\right)(1+\frac{K_m}{[s]})}$

  Only Vmax affected in the non-competitive model: $v_{max}^{app} = \dfrac{V_{max}}{(1+\frac{[I]}{K_{in}})}$

In these equations, v_o represents specific initial dechlorination rate (nmol of substrate dechlorinated/min/mg of protein), [S] is the substrate concentration (μM), K_m is the half saturation constant (μM), [I] is the inhibitor concentration (μM). K_{ic}, K_{iu} and K_{in} represent the competitive, uncompetitive and noncompetitive inhibition constant (μM), respectively. In the results presented below, the key parameter is the inhibition constant K_i (K_{ic}, K_{iu} or K_{in} depending on model) because this parameter provides an estimate of the concentration of the inhibitor where inhibition is significant, typically where dechlorination rates would be half the rate without inhibitor. Therefore, the lower the K_i, the greater is the inhibition. Some of the key findings from each of the four experiments listed above are summarized below.

Summary of results from Experiment #1: Effect of 1,1,1-TCA and 1,1-DCA on TCE, cDCE and VC dechlorination in three mixed cultures (OW, BDI and KB-1).
All three cultures contained *vcrA*, the gene encoding an RDase that catalyzes cDCE-to-VC dechlorination and VC-to-ethene dechlorination. Two KB-1 sub-cultures were also investigated: KB-1/*Dhc* maintained with VC and H_2 for 10 years contained only *Dhc* but not *Geobacter (Geo);* and KB-1/*Geo* is a sub-culture of KB-1, which contains a *Geobacter* sp. phylogenetically related (>99%) to *Geobacter lovleyi* strain SZ, a PCE-to-cDCE dechlorinator (150).

A summary of all culture, substrate, and inhibitor combinations tested ranked by K_i from lowest to highest, in both μM and μg/L (ppb) in shown in Table 1.2. Inhibition constants (K_i) are provided assuming the non-competitive model for all cell-free extract experiments to enable comparison between the different experiments. Examples of the model fit to experimental data are shown in Figure 1.15 (cell suspensions) and Figure 1.16 (cell-free extracts).
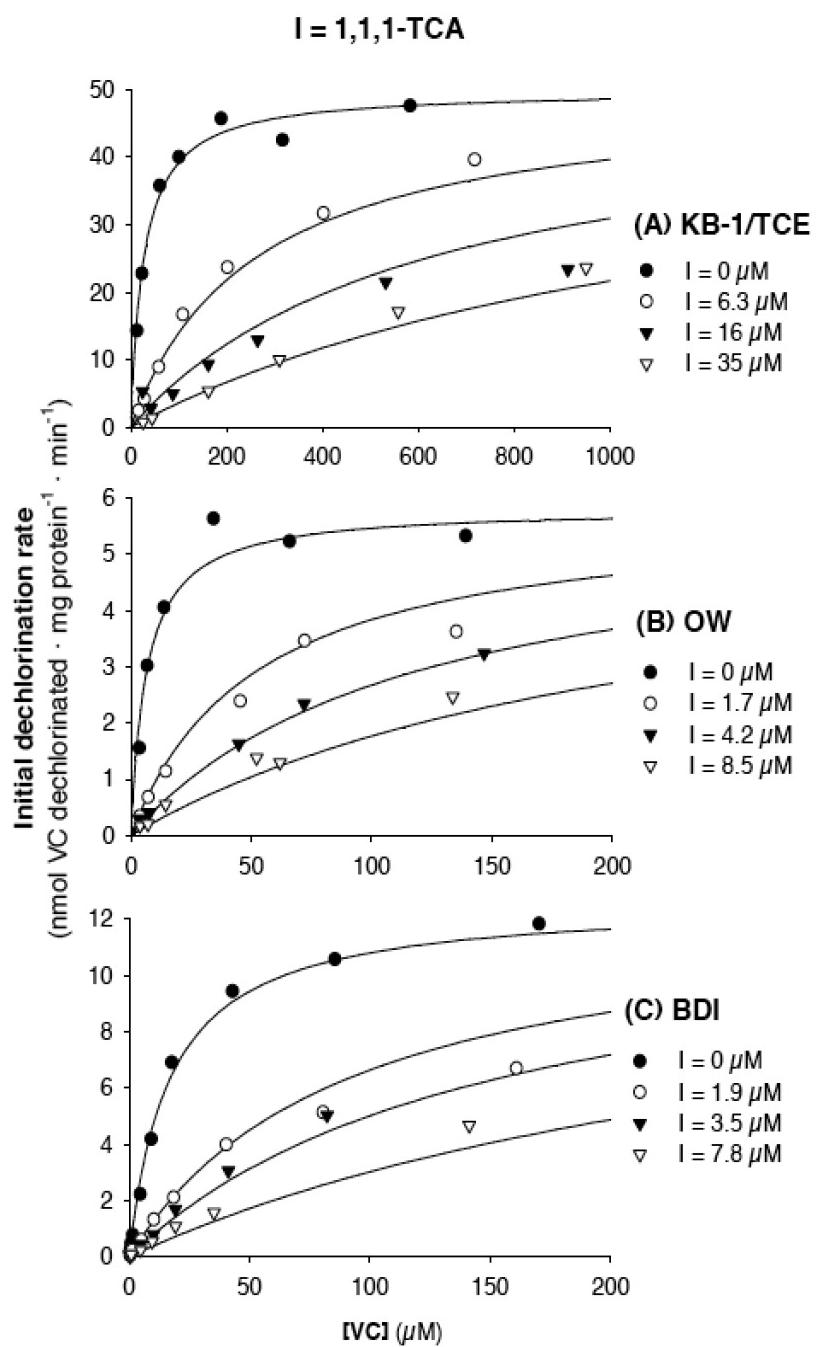
Figure 1.15. Kinetics of VC dechlorination by cell suspensions in the presence of increasing concentrations of 1,1,1-TCA. Three mixed cultures were compared: (A) KB-1, (B) OW, and (C) BDI. The 1,1,1-TCA concentrations for each assay series are indicated on each graph (I = inhibitor concentration in μM). Solid lines represent the best fit to each data set based on nonlinear regression using a competitive inhibition model (23).
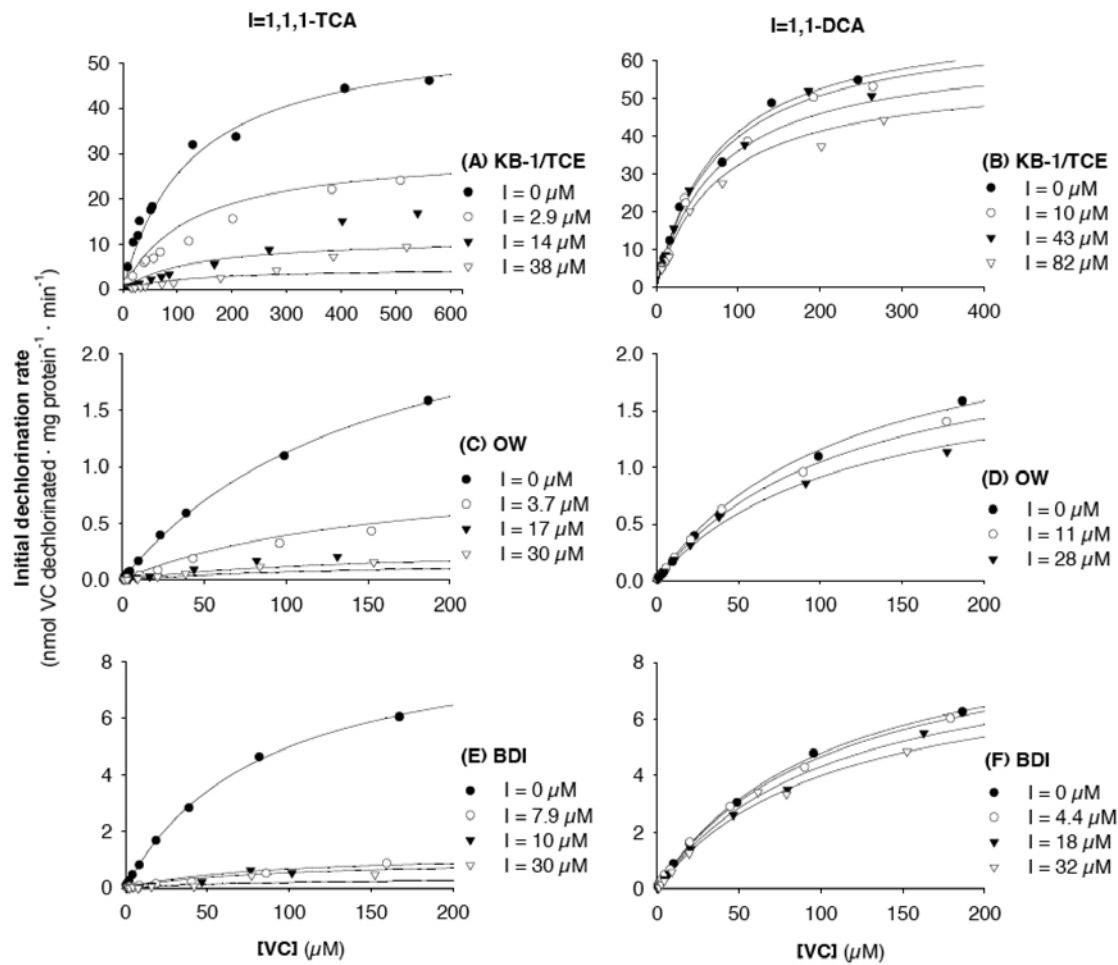
Figure 1.16. Kinetics of VC dechlorination by cell-free extracts in the presence of increasing concentrations of 1,1,1-TCA (left panels) or 1,1-DCA (right panels). Cell-free extracts were prepared from (AB) KB-1, (CD) OW, and (EF) BDI. Solid lines represent the best fit to each data set based on nonlinear regression using a noncompetitive inhibition model. I = inhibitor concentration in µM.

These data demonstrate that 1,1,1-TCA strongly inhibited VC dechlorination in both whole cell suspension and cell-free extract assays, suggesting that 1,1,1-TCA affects the VC reductase(s) associated with VC-to-ethene reductive dechlorination. Concentrations of 1,1,1-TCA in the range of 30-270 µg/L reduced VC dechlorination rates by approximately 50% relative to conditions without 1,1,1-TCA. 1,1,1-TCA also inhibited RDases involved in *cis*-DCE and TCE dechlorination (Table 1.2). In contrast, 1,1-DCA had no pronounced inhibitory effects on chlorinated ethene RDases (Figure 1.17), indicating that removal of 1,1,1-TCA via reductive dechlorination to 1,1-DCA is a strategy to relieve inhibition (21).

Table 1.2. Summary of all culture, substrate, and inhibitor combinations tested ranked by K_i from lowest to highest, in both µM and µg/L (ppb). Inhibition constants (K_i) are provided assuming the non-competitive model for all cell-free extract experiments enabling direct comparisons between these experiments.

				$K_i \pm 95\%CI$	
Culture	**Substrate**	**Inhibitor**	**Preparation**	µM	µg/L (ppb)
Cell Suspensions:				Competitive Model	
OW	VC	1,1,1-TCA	CS	0.2 ± 0.1	33 ± 11
BDI	VC	1,1,1-TCA	CS	0.4 ± 0.1	58 ± 13
KB-1	VC	1,1,1-TCA	CS	0.7 ± 0.2	100 ± 27
Cell-Free Extracts:				Non-Competitive Model	
KB-1	TCE	1,1,1-TCA	CFE	1.5 ± 0.6	210 ± 74
KB-1	VC	1,1,1-TCA	CFE	2.0 ± 0.3	270 ± 40
OW	VC	1,1,1-TCA	CFE	2.0 ± 0.4	270 ± 50
BDI	VC	1,1,1-TCA	CFE	2.1 ± 0.4	280 ± 50
KB-1/Dhc	TCE	1,1,1-TCA	CFE	2.2 ± 0.6	300 ± 75
KB-1/Geo	TCE	1,1,1-TCA	CFE	5.1 ± 2	690 ± 260
BDI	cDCE	1,1,1-TCA	CFE	5.5 ± 0.8	730 ± 110
KB-1	cDCE	1,1,1-TCA	CFE	19 ± 4	$2,500 \pm 600$
OW	TCE	1,1,1-TCA	CFE	40 ± 9	$5,300 \pm 1,200$
BDI	TCE	1,1,1-TCA	CFE	43 ± 17	$5,800 \pm 2,300$
OW	cDCE	1,1,1-TCA	CFE	86 ± 17	$11,500 \pm 2,300$
OW	VC	1,1-DCA	CFE	104 ± 24	$10,300 \pm 2,400$
BDI	cDCE	1,1-DCA	CFE	110 ± 18	$11,000 \pm 1,800$
OW	cDCE	1,1-DCA	CFE	130 ± 57	$13,000 \pm 5,600$
BDI	VC	1,1-DCA	CFE	162 ± 39	$16,000 \pm 3,700$
KB-1	VC	1,1-DCA	CFE	224 ± 111	$29,700 \pm 11,000$
KB-1	cDCE	1,1-DCA	CFE	830 ± 280	$82,000 \pm 28,000$
KB-1	TCE	1,1-DCA	CFE	No inhibition	No inhibition

Error values represent 95% confidence intervals

CS = Whole cell suspension

CFE = Cell-free extract

1,1,1-TCA inhibition of TCE dechlorination in consortium KB-1 did not fit well to the Michaelis-Menten model. Because both *Dhc* and *Geobacter* contribute to TCE dechlorination in consortium KB-1, we speculated that the poor model fit could be due to differences in enzyme affinities and catalytic activities between different TCE-dechlorinating RDases. To test this hypothesis, two sub-cultures of KB-1/TCE, one containing only *Dhc* (KB-1/VC) and the other predominantly *Geobacter* (KB-1/*Geo*), were tested in cell-free extract dechlorination assays. The *Geobacter* TCE reductase had a greater affinity to TCE (K_m=1.4 ±0.9 µM) compared to the *Dhc* RDase (K_m=180 ±40 µM) (21). This finding is consistent with previous calculations indicating that *Geobacter* is responsible for 80% of the TCE-to-cDCE dechlorination step in culture KB-1/TCE (28). However, dechlorination activities in both KB-1 sub-cultures were affected by the presence of 1,1,1-TCA. Interestingly, 1,1,1-TCA was less inhibitory to the TCE RDases in consortia BDI and OW suggesting differences between *Dhc* strains and/or *Dhc* RDases in terms of 1,1,1-TCA tolerances.

Implications for Chlorinated Ethene Reductive Dechlorination Activity at Sites with 1,1,1-TCA or 1,1-DCA as Co-Contaminant

The K_i values shown in Table 1.2 represent the inhibitor concentrations, at which the rate of dechlorination is half the rate compared to experimental systems without the inhibitor (this is mathematically true for the noncompetitive equation, and is true for the competitive equation as substrate concentrations decrease below K_m). The K_i values reported in Table 1.2 therefore provide useful guidelines to assess whether 1,1,1-TCA or 1,1-DCA concentrations will affect the reductive dechlorination of chlorinated ethenes. The data demonstrate that 1,1,1-TCA co-contamination should be a concern at all sites where practitioners seek to rely on microbial reductive dechlorination of VC to ethene as a remedial strategy. Fortunately, for all culture and chlorinated ethene combinations tested here, 1,1-DCA exerted low or negligible inhibition suggesting that the removal of 1,1,1-TCA via reductive dechlorination to its daughter products (1,1-DCA and CA) will relieve inhibition of chlorinated ethenes reductive dechlorination.
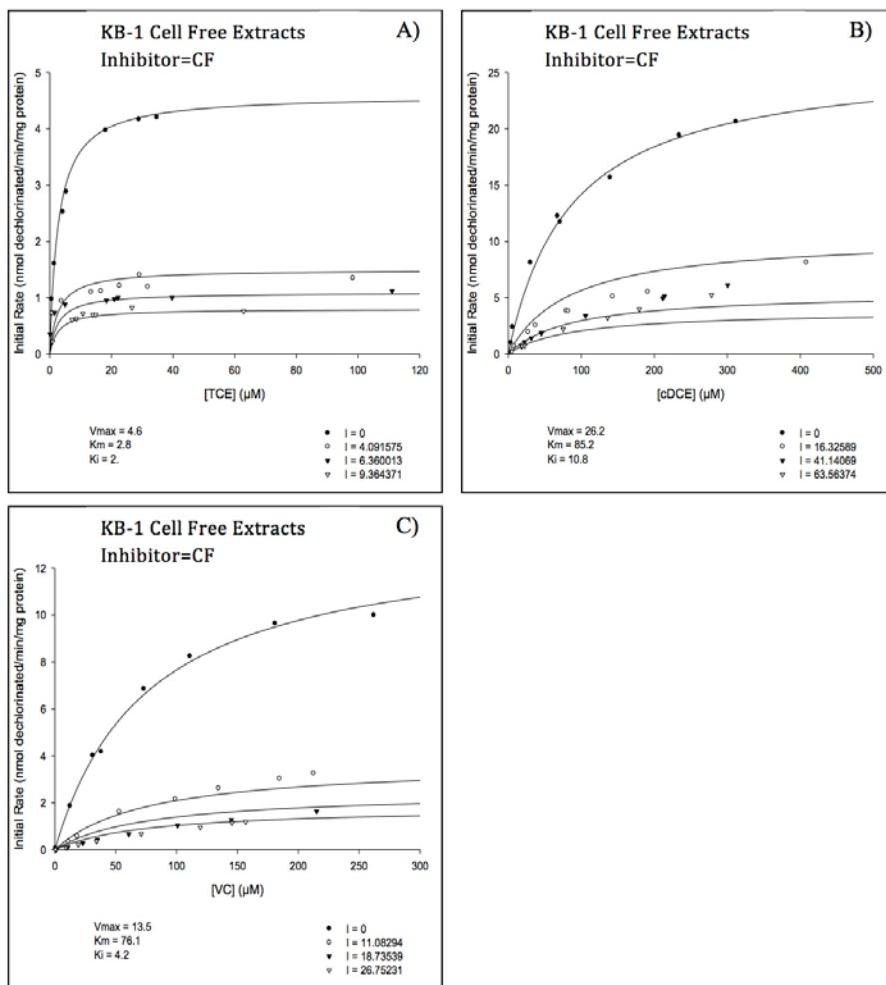
Figure 1.17. Kinetics of TCE (A), cDCE (B) and VC (C) dechlorination by cell-free extracts in the presence of increasing CF concentrations. Cell-free extracts were prepared from culture KB-1. Solid lines represent the best fit to each data set based on nonlinear regression using a noncompetitive mode. I is the CF concentration in µM. K_m and K_i are Michaelis-Menten and inhibition constants, respectively, and are reported in µM. V_{max} is the maximum initial dechlorination rate reported in nmol/min/mg protein.

This observation agrees with previous findings that dechlorination of TCE past cDCE and VC only proceeded when 1,1,1-TCA co-contamination was first removed by the addition of a 1,1,1-TCA-dechlorinating *Dhb*-containing mixed culture (45). The experiments described here used three different enrichment cultures that dechlorinated chlorinated ethenes to ethene. Since the diversity of *Dhc* populations carrying different RDases is not fully understood, it is certainly possible that VC-dechlorinating *Dhc* strains and *Dhc* RDases exist that differ in their responses to inhibitors such as 1,1,1-TCA.

Summary of Results from Experiments #2 and #3: Effect of chlorinated ethenes (TCE, cDCE and VC) on 1,1,1-TCA, 1,1-DCA and CF dechlorination in the ACT-3 culture. In these experiments, cell suspensions and cell-free extracts of the ACT-3 culture amended with the growth substrates 1,1,1-TCA, 1,1-DCA and CF were challenged with chlorinated ethenes to determine the extent, to which TCE, cDCE or VC inhibited reductive dechlorination. None of the chlorinated ethenes inhibited 1,1-DCA dechlorination in cell-free extract assays (Table 1.3), while 1,1,1-TCA and CF dechlorination were inhibited, particularly by VC (Table 1.3). This is consistent with our current knowledge that two different RDases dechlorinate 1,1-DCA and 1,1,1-TCA/CF. Interestingly, cDCE and particularly VC inhibited 1,1-DCA reductive dechlorination in whole cell assays (43), suggesting a general toxic effect on *Dhb* cells rather than a specific interaction with the RDase(s).

Table 1.3. Kinetic Parameters (V_{max}, K_m and K_i) for 1,1,1-TCA, 1,1-DCA and CF reductive dechlorination cell-free extracts of the ACT-3 culture in the presence of chlorinated ethenes. The best fit for all data was to an uncompetitive model, except in the case of 1,1-DCA as substrate, where no inhibition was observed. V_{max}, K_m and K_i values are shown with 95% confidence intervals.

Substrate	Inhibitor	V_{max} (nmol/min/mg)	K_m (μM)	K_i (μM)	Reference
1,1,1-TCA	TCE	102 ± 7	42 ± 6	42 ± 6	(43)
1,1,1-TCA	cDCE	86 ± 11	34 ± 10	126 ± 38	(43)
1,1,1-TCA	VC	73 ± 8	33 ± 8	35 ± 8	(43)
1,1-DCA	TCE	63 ± 6	461 ± 64	No Inhibition	(43)
1,1-DCA	cDCE	44 ± 5	289 ± 68	No Inhibition	(43)
1,1-DCA	VC	53 ± 3	396 ± 42	No Inhibition	(43)
CF	TCE	19 ± 0.63	22 ± 2.4	40 ± 3.1	(154)
CF	cDCE	23 ± 1.0	17 ± 3.4	6.7 ± 0.70	(154)
CF	VC	24 ± 1.0	23 ± 4.0	0.56 ± 0.052	(154)

A striking observation was the profound inhibition of CF reductive dechlorination by VC in cell-free extracts (Table 1.3), where the inhibition constant K_i was estimated to be as low as 0.5 µM. Interestingly, the inhibition constant for the same condition tested in cell suspension assays was at least an order of magnitude higher (~8 µM) (Table 1.4). These whole cell suspension results are consistent with the observed inhibition of CF dechlorination in growing cells (Figure 1.5), where CF dechlorination ceased when VC concentrations increased above 10 µM.

Table 1.4. Comparison of kinetic parameters (V_{max}, K_m and K_i) for 1,1,1-TCA and CF dechlorination between cell-free extracts and whole cell suspensions prepared from the ACT-3 culture in the presence of VC. The best fit for all data was to an uncompetitive model. V_{max}, K_m and K_i values are shown with 95% confidence intervals.

Preparation	Substrate	Inhibitor	V_{max} (nmol/ min/mg)	K_m (µM)	K_i (µM)	Reference
Cell free extract	1,1,1-TCA	VC	73±8	33±8	35± 8	(43)
Cell suspension	1,1,1-TCA	VC	3.0±0.3	13± 4	228±167	(43)
Cell free extract	CF	VC	24±1.0	23±4.0	0.56±0.052	(154)
Cell suspension	CF	VC	9.4±0.12	1.5±0.11	8.4±1.1	(154)

A closer comparison of the cell-free extract and whole cell suspension data (Table 1.4) revealed that inhibition was less pronounced in whole cell suspensions compared to cell-free extracts for both CF and 1,1,1-TCA, as shown by higher K_i values (by an order of magnitude) in whole cell suspensions. These data contrast observations in assays with *Dhc*-dominated cultures such as KB-1 where cell suspensions had somewhat lower K_i values than cell-free extracts (Table 1.2). These findings suggest that the membranes of *Dhb*, which are structurally quite different from those of *Dhc*, may offer some protection to the cell.

**Implications for Reductive Dechlorination of 1,1,1-TCA and CF at Sites Co-
Contaminated with Chlorinated Ethenes**

Inhibition constants reflect the affinity of inhibitors for their target and indicate the
inhibitor concentration that causes inhibition. In the context of bioremediation, a
comparison of half velocity constants (K_m) to inhibitor constants (K_i) is a measure of the
relative potency of a co-contaminant for inhibiting a transformation reaction of interest.
The lowest inhibition constants and most potent inhibition were observed in cell-free
extracts with CF or 1,1,1-TCA as the substrate in the presence of VC as inhibitor.
However, the data from whole cell suspension assays using intact cells are more
representative of in situ conditions.

During 1,1,1-TCA dechlorination, whole cell suspension measurements revealed TCE
and VC inhibition constants in the range of 225-250 µM, an order of magnitude greater
than the apparent K_m in cell-free enzyme assays. cDCE was consistently less inhibitory
towards 1,1,1-TCA dechlorination in both whole cell and cell-free extract assays.
Therefore, 1,1,1-TCA dechlorination will proceed in the presence of VC and TCE as long
as their concentrations are below approximately 200 µM (26 mg/L for TCE). At higher
TCE concentrations, given the uncompetitive nature of the observed inhibition, 1,1,1-
TCA dechlorination rates would be predicted to decline by a factor of approximately
$(1+[I]/K_i)$. For example, at a TCE concentration of 45 mg/L (340 µM), which is not
uncommon at sites with source zones, and assuming a K_i of about 225 µM, the rate of
1,1,1-TCA dechlorination would be predicted to be about 2/5 the rate in the absence of
TCE. When 1,1-DCA dechlorination was measured in whole cell suspensions, both the
K_m and K_i were in the range of 80-200 µM (43). Therefore, it would be expected that the
presence of chlorinated ethenes in this range would significantly impact the reductive
dechlorination of 1,1-DCA to CA, which was previously observed in this mixed culture
(45). In this previous study, cDCE and VC accumulated up to 380 and 140 µM,
respectively, and dechlorination of 1,1-DCA to CA ceased entirely until the chlorinated
ethenes had been completely reduced to ethene. VC had a profound inhibitory effect on
CF reductive dechlorination, even in whole cell suspensions, with a K_i of about 8.4 µM
or 500 µg/L. Therefore, at a concentration of 2x K_i (1 mg/L VC), the rate of CF

dechlorination would be 1/3 the rate without VC, and at a concentration of 9x K_i (4.5 mg/L), the rate would be 1/10 the rate without VC.

Summary of results from Experiment #4: Effect of CF on TCE, cDCE and VC dechlorination by culture KB-1. To better understand the inhibition observed in cultures exposed to CF (Figure 1.2), and to elucidate the interactions observed when the ACT-3 and KB-1 cultures were mixed in an attempt to dechlorinate both CF and TCE (Figure 1.6), inhibition constants were determined in culture KB-1 exposed to varying concentrations of CF (Figure 1.17).

Table 1.5. **Kinetic Parameters (V_{max}, K_m and K_I) for chlorinated ethene dechlorination in cell-free extracts from the KB-1 culture in the presence of CF and 1,1,1-TCA.** Kinetic Parameters (K_m, V_{max} and K_i) for chlorinated ethene reductive dechlorination in KB-1 cell-free extracts in the presence of CF or 1,1,1-TCA. Data shown are for the best-fit noncompetitive model.

Preparation	Substrate	Inhibitor	V_{max} (nmol/min/mg)	K_m (µM)	K_i (µM)	Reference
Cell free extract	TCE	CF	4.6±0.11	2.8±0.29	2.0±0.11	(154)
Cell free extract	cDCE	CF	26±4.5	85±13	11±0.94	(154)
Cell free extract	VC	CF	14±0.47	76±6.6	4.2±0.22	(154)
Cell free extract	TCE	1,1,1-TCA	82±13	40±19	1.5±0.6	(21)
Cell free extract	cDCE	1,1,1-TCA	91±7	86±22	19±4	(21)
Cell free extract	VC	1,1,1-TCA	49±4	83±15	2.0±0.3	(21)

In cell-free extracts, CF inhibited chlorinated ethene reductive dechlorination to a similar extent as was observed with 1,1,1-TCA (Table 1.5). Whole cell suspensions showed similar trends. However, the inhibition constants measured in whole cell suspensions and in cell-free extracts did not reflect well the data observed in growing KB-1 cultures (Figure 1.1) where cultures were very slow to overcome exposure to CF, suggesting that CF leads to cell death. CF is possibly irreversibly inhibiting other cellular processes, which would lead to a more significant loss in dechlorination activity than that predicted by the inhibition constants. These observations are relevant for bioremediation and warrant further investigation.

Implications for Chlorinated Ethene Reductive Dechlorination Activity at Sites Co-Contaminated with CF and 1,1,1-TCA

CF and 1,1,1-TCA both inhibit chlorinated ethene reductive dechlorination to a similar extent in cell-free extract experiments. Therefore, either compound at sites contaminated with chlorinated ethenes can severely hamper bioremediation based on *Dhc* reductive dechlorination activity. A major difference between 1,1,1-TCA and CF is not their effect on chlorinated ethene dechlorination, but the fact that their own dechlorination is inhibited in the presence of chlorinated ethenes. 1,1,1-TCA dechlorination is significantly less inhibited by TCE and VC than CF dechlorination, and therefore 1,1,1-TCA dechlorination can proceed to 1,1-DCA, allowing TCE, cDCE and VC to be dechlorinated to ethene, thereby relieving inhibition of 1,1-DCA dechlorination associated with VC. In the case of co-contamination with CF, the scenario is different because CF dechlorination is more strongly inhibited by TCE and VC. Therefore, CF dechlorination does not go to completion, causing dechlorination of TCE and cDCE to stall at VC, and accumulation of VC stops further CF dechlorination; thus all further dechlorination is suspended. Of course, these effects are highly concentration-dependent, and the inhibition constants provide guidelines to threshold concentrations where effects are likely to become important considerations. Of course, differences between *Dhc* strains and/or *Dhc* RDases in terms of tolerances to inhibitors may exist, which is supported by the observation that 1,1,1-TCA had a decreased inhibitory effect on the *Dhc*-containing consortia BDI and OW.

Task 2: Identifying Novel Biomarker Genes

Several independent approaches were used to assign function to genes encoding RDases. First, three complimentary microarrays were used to identify reductive dechlorination biomarker genes and assign function to *Dhc* RDase genes.

- RDase gene array
- *Dhc* pan-genome array
- KB-1 metagenomic array

The RDase gene array was designed using sequence information of RDase genes known to be responsible for specific dechlorination reactions (e.g., *tceA, bvcA, vcrA*), as well as for any RDase gene sequences and genes associated with the reductive dechlorination process that existed in the GenBank database at the time of array construction. To better understand global *Dhc* metabolic processes, a pan-genome-scale hybridization array was designed with a corresponding metabolic network and a constraint-based metabolic model of *Dhc*. The pan-genome array was constructed from the complete genomes of *Dhc* strain CBDB1, strain 195, strain BAV1, and strain VS. Finally, to examine the performance of the dechlorinating consortium KB-1, a KB-1 metagenomic array was constructed from total genomic DNA of the KB-1 mixed culture.

In addition, a new application of Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) was developed to measure reductive dechlorination activity in gels following electrophoretic protein separation. Subsequent liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) identified peptides of the active RDase(s) in the gel slices. By means of the peptide sequence information, genes encoding RDases were identified.

RDase Gene Array

RDase genes responsive to chlorinated compounds of interest (e.g., chlorinated ethenes) are potential biomarkers for monitoring reductive dechlorination processes. The RDase array included probes that targeted RDase genes that were identified in public databases (e.g., GenBank). Also included were probes targeting *Dhc* hydrogenase genes, genes involved in DNA replication, putative phage genes identified on *Dhc* genomes, and 16S rRNA genes of relevant organohalide-respiring bacteria. Additional RDase genes were identified in clone libraries established with available degenerate primers targeting RDase operon-conserved features (76). For most target genes, three specific probes were designed using the *OligoArray 2.0* program. The probes consisted of 30-50-mer oligonucleotides spotted onto epoxy coated glass slides (i.e., spotted oligo array). A C6-amino linker added at the 5' end of the oligos covalently linked the probes unidirectionally away from the microarray surface, allowing greater access to the labeled

target during hybridization. The RDase arrays were printed at Michigan State University's Research Technology Support Facility in duplicate 8-pin arrays and each unique probe is represented twice on the slide. As new putative RDase genes were added to public databases, the RDase array was updated accordingly. The numbers of probes targeting gene categories relevant for the reductive dechlorination process are summarized in Table 2.1. A probe targeting an *Arabidopsis* gene was added as an internal control and for signal normalization.

Table 2.1. Features of the 1st and 2nd generation RDase gene arrays.

Target	# of probes			
	1st Generation array		2nd Generation array	
	# of Target Sequences	# of probes	# of Target Sequences	# of probes
RDase genes (*Dhc*)	234	662	265	748
RDase genes (other dechlorinators)	69	198	76	217
Hydrogenase genes	104	297	109	312
16S rRNA genes	38	114	38	114
Phage-related (*Dhc*)	55	157	102	296
Arabidopsis thaliana chlorophyll synthetase gene	1	3	1	3
Total	501	1,431	591	1,690

Initial testing of the RDase microarray included verification of probe specificity using genomic DNA from consortium BDI, DNA from the *Dhc*-containing 1,2-DCP-dechlorinating cultures KS and RC (85, 126), and DNA from the *Dhc* isolates BAV1 and CBDB1 (3, 50). Optimal slide hybridization was at 50°C with a ratio of cyanine fluorescent molecules Cy5 and Cy3 at an aa-dUTP to dTTP ratio of 2:3. Additional experiments using 2-250 ng of consortium BDI DNA and 500-4,000 ng of *Dhc* strain GT genomic DNA determined that levels of 10^5-10^7 copies of the target gene are required to achieve detection.

Further experiments with the 2nd generation RDase array demonstrated the probes' specificity using *Dhc* pure culture genomic DNA and genomic DNA from a TCE-fed BDI culture. To further validate probe specificity, RDase gene fragments (*bvcA, vcrA, tceAB*) were cloned into the pCR2.1 TOPO TA vector (Invitrogen). These plasmids were aminoallyl-labeled using the exo-Klenow fragment, labeled with one Cy dye, purified and hybridized to the RDase array. The experiments demonstrated that all three oligos designed for these target genes yielded reproducible signal intensities and were specific to their intended targets (e.g., only *bvcA*-like genes with >99% sequence similarity yielded a positive signal when the plasmid containing the *bvcA* gene from *Dhc* strain BAV1 was hybridized to the array) (Figure 2.1).
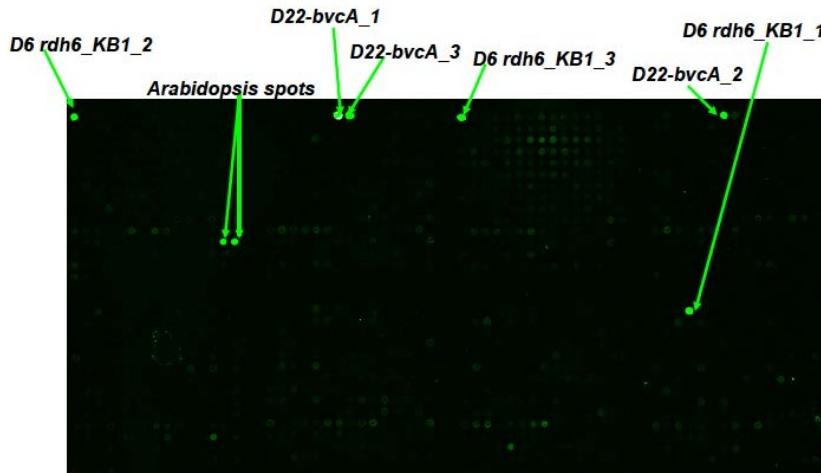
Figure 2.1. Interrogation of the RDase array with *bvcA* amplicons. A *bvcA* gene fragment was amplified from the pCR2.1-TOPO vector containing the *bvcA* gene using the degenerate primers B1R and RR2F (76), and the amplicons were labeled and hybridized to the array. The underscore " _ " and number after a gene name denotes the oligo number (D22-*bvcA*_1, D22-*bvcA*_2, D22-*bvcA*_3). The *Arabidopsis* spike (positive control) showed a strong hybridization signal. The results demonstrate that only *bvcA*-like genes with >99% sequence similarity gave generated signals with all three probes, and included the *bvcA* gene of *Dhc* strain BAV1 as well as rdhA6, which is a *bvcA*-like gene present in the mixed culture KB-1 (99% sequence similarity to bvcA; 1,646 of 1,653 nucleotides are identical).

Another experiment used the degenerate primers pair B1R and RR2F (76) to amplify RDase genes from consortium BDI, and the resulting amplicons were used to interrogate the RDase gene array. This degenerate primer pair targeted a sequence stretch encoding the conserved RRXFXK motif near the N-terminus of the RDase and the sequence

encoding the WYEW motif of the adjacent B protein. This primer set has been used to obtain and clone RDase gene sequences from *Dhc* pure and enrichment cultures (76). Amplicons obtained with primer set B1R and RR2F from the dechlorinating consortium BDI were labeled and hybridized to the array, and the results are displayed in Figure 2.2. The amplicons only hybridized to RDase gene probes and no signals were obtained for non-RDase genes (Table 2.2). These findings corroborate the specificity of the RDase gene-targeted probes. The evaluation of the hybridization signals revealed that the B1R and RR2F primer pair failed to amplify all RDase genes present in the genomes of the *Dhc* strains present in consortium BDI, which yielded the genomic template DNA for PCR amplification. For example, this primer set did not amplify the *tceA* gene.
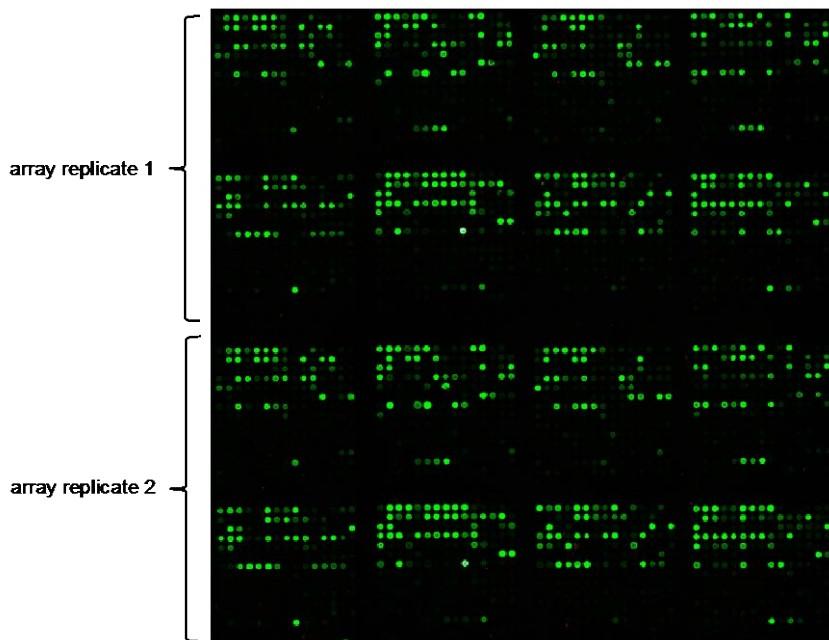
Figure 2.2. Amplicons obtained with the degenerate, RDase gene-targeted primer pair B1R-BB2F and hybridized to the 2nd generation RDase array. PCR products amplified with primer set B1R and RR2F from the dechlorinating consortium BDI were labeled and hybridized to the array.

Hybridization with genomic DNA from *Dhc* strain BAV1 and *Geobacter lovleyi* strain SZ (138) were used to evaluate the best method for microarray data analysis. To bioinformatically determine the specificities of all the probes with their target sequences in relationship to the experimentally obtained microarray signal intensities, the BLAST

score ratio (BSR) was calculated as described (117). A BSR of 83% was selected as the signal threshold for recognizing probes that provided hybridization signals greater than the background signal (116, 117). A similar cross hybridization sequence identity cutoff of 85% was determined for 50-mer probes in other spotted microrray applications (55, 117). Furthermore, different methods for defining gene presence/absence were tested. In microarray experiments, the signal-to-noise (SNR) ratio can define a positive signal by differentiating it form the background noise. Two different SNR calculations are described in the literature (55, 88, 89) and different signal to noise thresholds are arbitrarily assigned. Evaluation of the two different SNR methods (based on a BSR of 83%) and five different SNR thresholds (1 to 5) indicated that the SNR method described by Loy and Bodrossy (88) with a signal threshold of 3 was appropriate to define gene presence/absence in the RDase hybridization signal datasets. These parameters yielded the least false positives (FP) and false negatives (FN) and the highest signals (85%) from "good" probes (GP).

Application of the RDase gene array. The RDase array was applied to three independent experimental systems to test its utility to detect RDase genes or transcripts, as well as other genes/transcripts associated with the reductive dechlorination process.

Example 1. *RDase gene transcription in consortium BDI grown with PCE versus TCE as electron acceptor.* cDNA was generated from RNA obtained from consortium BDI grown under different conditions with the goal to identify genes differentially transcribed, suggesting their involvement in specific dechlorination reactions. Triplicate 160-mL serum bottles containing 100 mL of BDI culture were spiked with 40 µmoles of PCE, TCE, *cis*-DCE or VC as electron acceptors. Three hours after the chlorinated substrate was spiked into the cultures total RNA was extracted and reverse transcribed. Aminoallyl-labeled cDNAs were coupled with cyanine dyes (Cy5 and Cy3) and hybridized to duplicate RDase array slides. The slides were scanned with an Axon 4000B Scanner. A volcano plot depicts the fold change in transcription vs. significance (P-value) for t-test results (Figure 2.3). Among the prominent genes differentially transcribed when TCE was used as electron acceptor were the *Dhc* gene annotated as

formate dehydrogenase (H109-VS-Fdehy0790) and genes that corresponded to RDase B genes (e.g., *tceB*) encoding the putative RDase anchoring proteins, suggesting that transcripts of these genes could provide prospective biomarkers for actively dechlorinating *Dhc* cells.
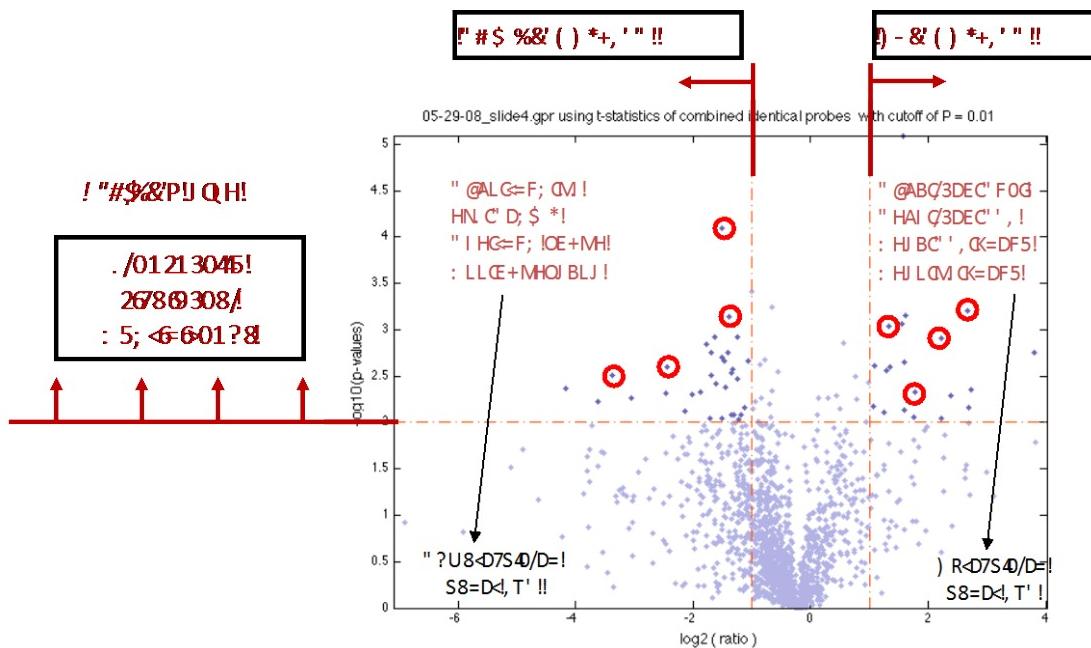
Figure 2.3. Volcano plot showing genes differentially transcribed in consortium BDI with PCE versus TCE provided as electron acceptor. The volcano plot shows p-value versus fold change in transcription level. The x-axis is the fold change value and the y-axis is the -log10 p-value. Using the absolute 2-fold change and p-value 0.01 as the threshold cutoff, in the upper left and upper right are genes that are differentially transcribed. Some genes marked with a circle are potential biomarker candidates that are differentially transcribed when TCE is supplied as electron acceptor.

Example 2. *RDase gene detection in a polychlorinated biphenyl- (PCB-) dechlorinating consortium.* The hybridization of genomic DNA extracted from a PCB-dechlorinating culture (12, 13) identified 27 different RDase genes within the mixed culture. RDase gene clone libraries established with primer pair B1R and RR2F using genomic DNA obtained from an earlier PCB-dechlorinating enrichment yielded 25 distinct RDase gene sequences, 20 of which were also identified with the RDase array. The absence of the five previously detected RDase genes likely reflects the loss of organisms carrying RDase genes due to the continued enrichment with specific PCB congeners. These results demonstrate the utility of the RDase array to detect known RDase genes in reductively dechlorinating enrichment cultures.

Example 3. *Application of the RDase array to environmental samples.* To demonstrate the utility of the RDase array as a monitoring tool, groundwater samples were collected from a well at a PCE/TCE-contaminated site in Georgia, USA, prior to bioremediation, and at three time points following bioaugmentation and biostimulation over the course of 7 months. Total DNA was extracted from each groundwater sample, and the same amounts of labeled DNA were hybridized to 2nd generation RDase arrays. Interrogation of the array with samples prior to bioremediation treatment did not reveal the presence of RDase genes implicated in the reductive dechlorination of chlorinated ethenes (i.e., *pceA*, *tceA*, *bvcA*, *vcrA*) (Figure 2.4, Sample T0). This analysis demonstrated that *Dhc* strains carrying these RDase genes increased in abundance (i.e., growth occurred) in response to biostimulation. Among the genes that increased in abundance after biostimulation were RDase genes with assigned function such as the *pceA* gene implicated in PCE/TCE-to-cDCE reductive dechlorination, the *tceA* gene responsible for TCE-to-VC reductive dechlorination, and the *vcrA* and *bvcA* genes implicated in cDCE and VC reductive dechlorination.

T_0   **T_1**   **T_2**   **T_3**

Before treatment   **After treatment**   **2 months after treatment**   **5 months after treatment**
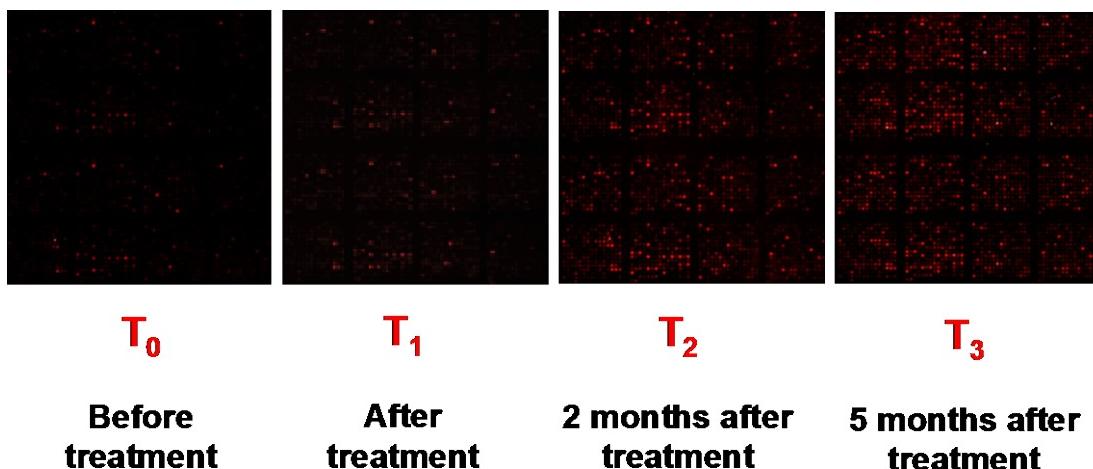
Figure 2.4. Microarray visualization of DNA samples from a chlorinated solvent-contaminated site prior to bioremediation and at three time points following biostimulation/bioaugmentation with a dechlorinating consortium. Identical DNA amounts extracted from groundwater from the same monitoring well were labeled with the fluorescent dye Cy5 and hybridized to the RDase array. After washing, the slide was scanned with a laser at a wavelength of 635 nm and red fluorescence was recorded. A red signal indicates the presence of a target gene in the sample and the color intensity can be used to infer relative abundance.

Figure 2.5 depicts the relative increase in the abundance of the *pceA*, *tceA*, *vcrA* and *bvcA* genes following bioremediation treatment as determined with the RDase microarray.
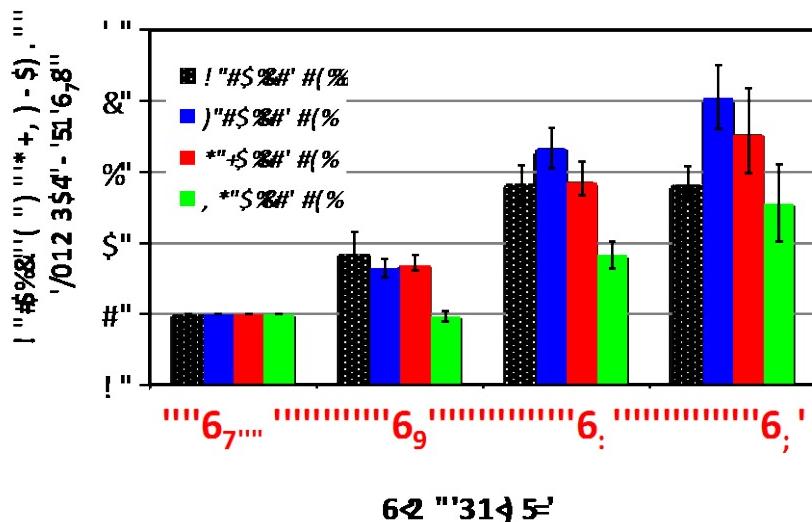
Figure 2.5. Relative RDase gene abundances inferred by the RDase microarray from DNA samples from a chlorinated solvent contaminated site prior to treatment and at three time points following biostimulation and bioaugmentation. The intensity values (signals) generated by probes targeting RDase genes with assigned function (i.e., *pceA*, *tceA*, *vcrA* and *bvcA*) were individually grouped and averaged. For example, the microarray included probes targeting the *pceA* genes of *Sulfurospirillum*, *Dhc*, *Desulfitobacterium* and *Geobacter*. Error bars represent the standard error.

These data demonstrate the feasibility of using the RDase microarray for monitoring *Dhc* strains with known suites of RDase genes at sites undergoing bioremediation. Since *Dhc* strains can only grow with the chloroorganic contaminants as electron acceptors and hydrogen as electron donor, detecting fewer target genes or diminishing color intensity signals without reaching contaminant cleanup goals may suggest that *Dhc* activity is limited, e.g., by electron donor availability. Although this example demonstrates that microarrays could be applied as a monitoring tool to determine if electron donor additions, or other amendments (e.g., pH adjustment) are needed, and thus support decision-making, limitations were noted. Key shortcomings of the microarray approach for monitoring genes of interest in environmental samples are discussed at the end of the microarray section.

Dehalococcoides Core/Pan-Genome Array

A core/pan-genome array was designed to be applicable to both sequenced and unsequenced *Dhc* genomes. Thus, the core/pan-genome array promises to identify *Dhc* genes of unknown strains (i.e., *Dhc* strains lacking genome information) expected to be present in environmental samples. The array design considered all available *Dhc* genome sequences to find probes that would apply to a majority of known *Dhc* genes. The design efforts were based on the sequenced genomes of *Dhc* strains 195, CBDB1, BAV1, VS, and GT, and partial sequence information of the *Dhc* strains present in consortium KB-1.

Pan-Genome Metabolic Model. The categorization of all the genes in known *Dhc* strains (i.e., the *Dhc* pan-genome) enabled the creation of a metabolic model for *Dhc*. The *Dhc* pan-genome consisted of 1,118 core genes (shared by all known *Dhc* strains), 457 dispensable genes (shared by some strains) and 486 unique genes (found in only one genome) (71). Metabolic genes were identified from the pan-genome gene set by rigorous sequence comparison to public databases and the published literature. This analysis identified 549 metabolic genes that encoded 356 proteins catalyzing 497 gene-associated reactions. Of these 497 reactions, 477 were associated with core metabolic genes, 18 with dispensable genes and two with unique genes found in only one genome. These metabolic genes and associated reactions were used to develop a pan-genome-scale constraint-based *in silico* metabolic model of *Dhc* (71). The model provides insights into *Dhc's* metabolic limitations, low growth rates and energy generation. The model provides a framework to anchor and compare disparate experimental data, such as the core/pan-genome microarray data described below. Further, the model provides insights and quantitative data on the physiological impact of "incomplete" pathways, such as the TCA-cycle, CO_2 fixation and the cobalamin biosynthesis pathway (71).

Pan-Genome Microarray. The pan-genome microarray provides coverage of all *Dhc* core genes as well as strain-specific genes while optimizing the potential for hybridization to closely related, environmental *Dhc* strains (66). The pan-genome probe set was compared to probe sets designed independently for each of five *Dhc* isolates. The pan-genome probe set demonstrated better predictability and higher detection frequency of *Dhc* genes than strain-specific probe sets on non-target *Dhc* strains with <99% average

nucleotide identity. An *in silico* analysis of the expected probe hybridization against the subsequently released *Dhc* strain GT genome and additional KB-1 metagenome sequence data indicated that the pan-genome probe set performs more robustly than the combined strain-specific probe sets in the detection of genes not included in the original design. Thus, the pan-genome microarray represents a highly specific, universal tool for the detection and characterization of *Dhc*. This array is a useful common platform for *Dhc*-focused research, allowing meaningful comparisons between microarray experiments regardless of the specific *Dhc* strain(s) examined (66).

The pan-genome probe set used in these microarrays was designed using an innovative program called ProDesign (33). In order to test the utility of the pan-genome probe set developed using ProDesign for experimentation on specific *Dhc* strains, the Agilent eArray system (with all default parameters for bacterial genomes) was used to design probes for the complete gene complement of each individual genome sequence (or partial genome sequence, in the case of KB-1). *In silico* comparison of the individual genome probe sets and the combined pan-genome probe set indicated that the pan-genome probe set provided an acceptable level of coverage for the individual genomes, and should function equally well for single genome examinations, while additionally providing an universal probe set that can be directly compared between different laboratories. This was in fact shown when the probes were tested with a variety of different DNA samples (Figure 2.6). The core/pan-genome array design is available from Agilent Technologies and arrays can be purchased without restrictions.

Shotgun Array of Mixed Culture KB-1

The RDase array and the pan-genome array do not include any probes for the non-*Dhc* populations relevant for supporting the reductive dechlorination process. Therefore, a shotgun metagenomic microarray from DNA fragments generated from the KB-1 consortium was constructed (152). In this way, probes targeting organisms other than *Dhc* would be represented on the array and could be interrogated. *Dhc* strains grow best in mixed cultures, relying on non-dechlorinating members to provide essential nutrients and to maintain reducing conditions.

It should be noted that these arrays were first conceived in 2003, when genome sequencing was expensive and not readily accessible. This type of array is now superseded by oligonucleotide arrays where probes are generated to known sequences retrieved from genomes or metagenomes, or by RNA-Seq technologies that circumvent arrays all together. Nevertheless, the experiments conducted on these arrays provided an effective screening tool to identify potential biomarker targets for follow-up experiments. In particular, these arrays pointed to interesting gene expression results under starvation conditions. These are relevant for understanding the fate of *Dhc* cells in the environment, where starvation is a frequent occurrence.
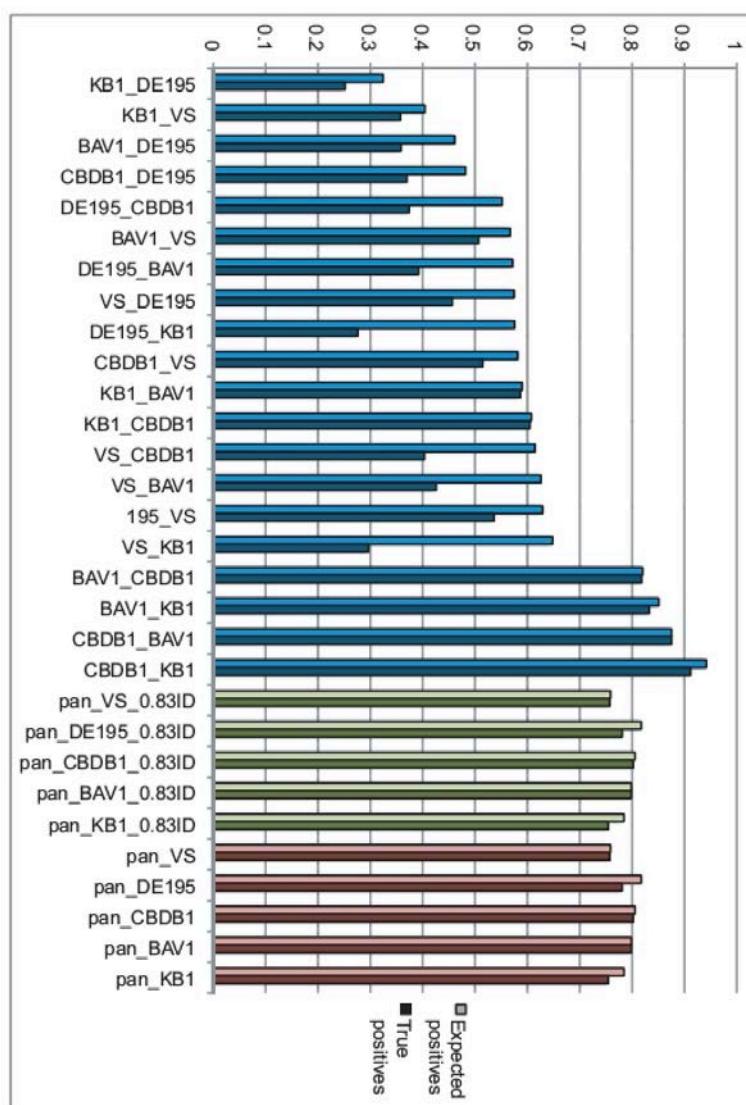
Figure 2.6. Proportion of genes per genome covered by probe sets. Column names are in the format probe set_DNA sample hybridized. Light bars indicate the proportion of genes predicted to be detected, while dark bars indicate the actual proportion of genes detected. Predicted probe hybridization was based on either an 83% Blast score ratio (strain-specific probes in blue, pan-genome probes in green), or, for the pan-genome probes, on gene presence within cd-hit-est clusters, as designed (red). Detected positives were based on a normalized fluorescence signal threshold of 1.46×10^4. Pan-genome probe set data where statistical measures were based on an expected hybridization pattern are presented in red (66).

The KB-1 metagenomic microarrays were constructed

from total community genomic DNA of the KB-1 consortium. Each array contained

19,200 spots (152). The clones used to make the spotted fragments were sequenced at

the Department of Energy's Joint Genome Institute (DOE-JGI) in Walnut Creek, CA,

with additional sequencing performed at The Atlantic Genome Center (TAGC, Halifax,

NS, Canada). Putative functions and phylogenies were ascribed to the spots based on

their sequences. We investigated gene transcription during VC dechlorination and during

starvation (no chlorinated electron acceptors provided) in culture KB-1 (153). In both

treatment conditions, methanol was amended as an electron donor. During VC

dechlorination, *Dhc* genes involved in transcription, translation, energy conservation, as

well as amino acid and lipid metabolism and transport were over-represented in the

transcripts as compared to the average *Dhc* gene (Figure 2.7).
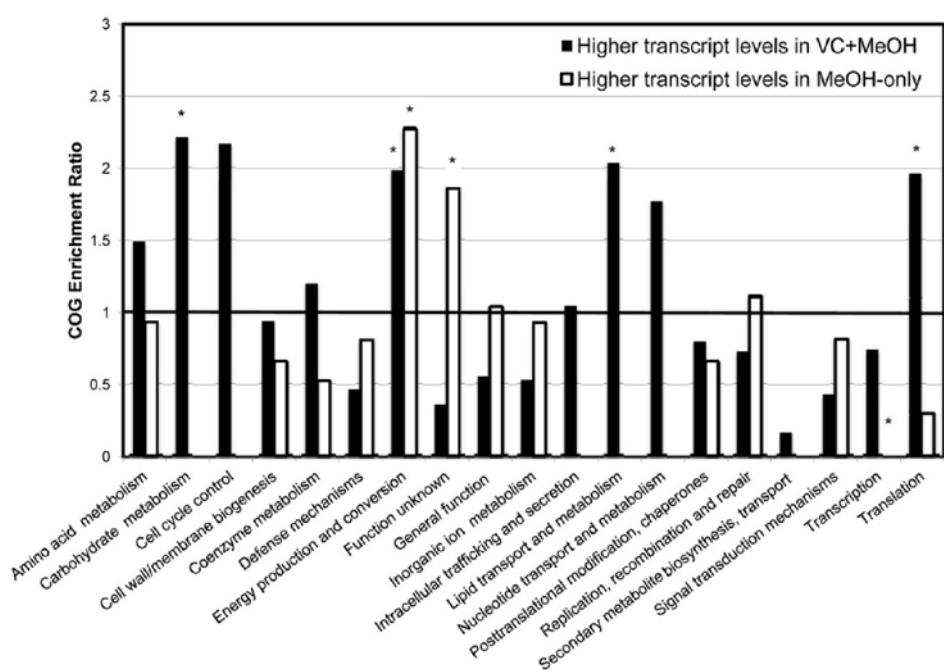
**Figure 2.7. Representation of clusters of orthologous group (COG) categories among
Dhc genes exhibiting different transcript levels between treatments.** On the x-axis are
COGs as defined by the eggNOG database. On the y-axis is an enrichment ratio
representing the relative proportion of each COG category in the sequenced spots compared
to the average COG abundance in *Dhc* genomes according to the equation: enrichment ratio
= proportion of COG X in *Dhc* sequences with higher transcript levels/proportion of COG X in
Dhc genomes. A ratio of 1 indicates that there is the same proportion of that COG in the
sequenced spots as in an average *Dhc* genome. COGs that have statistically significant
enrichment ratios are marked with an asterisk. P values were calculated using a
hypergeometric distribution.

KB-1 *rdh*A14 (*vcrA*) was the only RDase gene with higher transcript levels during VC degradation, while multiple RDase genes had higher transcript levels in the absence of VC (153). Four putative RDase genes were identified in the absence of VC (i.e., starved for electron acceptor): KB-1 *rdhA5, rdhA1, rdhA12* and *rdhA13*. Specifically, the transcription level of KB-1 *rdhA5* was among the highest in the methanol-only treatments. The increased expression of *rdhA5* in the absence of VC is consistent with the expression profile of its ortholog in *Dhc* strain 195 (DET1545), which is upregulated in stationary phase (72). These data were supported by subsequent proteomic analyses of the culture in BN-PAGE experiments where peptide sequences matching *rdhA5 and rdhA1* sequences were identified (142). Numerous hypothetical genes from *Dhc* also had higher transcript levels in methanol-only (starved) treatments and indicate that many uncharacterized proteins are involved in cell maintenance in the absence of chlorinated electron acceptors. The most highly transcribed genes have been tabulated and several of these have been cloned and heterologously expressed in *E. coli* for future investigations and development of biomarkers (153)

Transcripts from non-Dhc microorganisms and corrinoid synthesis and salvaging genes. Transcripts from *Spirochaetes, Chloroflexi, Geobacter* and methanogens demonstrate the importance of non-*Dhc* microorganisms, and sequencing of identified shotgun clones provided sequence information for follow-on studies (153). Because corrinoid is an essential cofactor for *Dhc* RDases, transcription of genes involved in corrinoid biosynthesis was investigated. Many *Dhc* and non-*Dhc* genes involved in corrinoid biosynthesis were transcribed and differentially expressed during dechlorination. Overlaying the response of these genes to a diagram of the cobalamin biosynthesis pathway illustrated that many *Dhc* genes in the latter part of the pathway were upregulated and/or transcribed (Figure 2.8). In addition, genes involved in corrinoid transport were transcribed, supporting the salvaging of corrinoids from the environment. Moreover, transcription of genes involved in the early stages of porphyrin ring formation and methylation suggested *Methanoregula, Syntrophus,* and *Geobacter* as possible producers of corrinoid precursors (152, 153). The investigation of the roles of the non-*Dhc* community members in mixed cultures was also the objective in a comparative

metagenomic analysis described in the next section (63, 64). This study also highlighted

the potential role of methanogens in provision of corrinoid precursors.
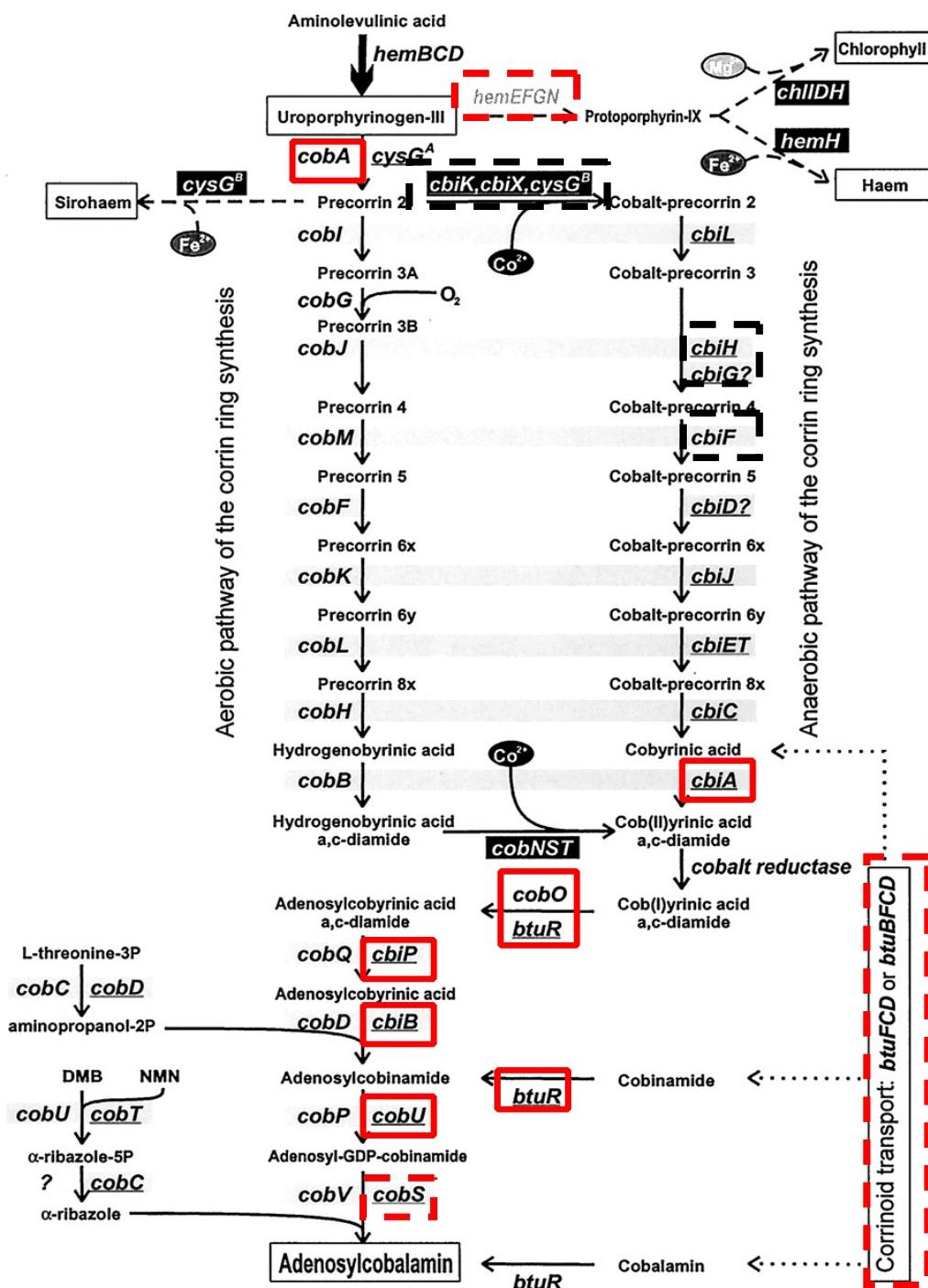
Figure 2.8. **Biosynthetic pathways for adenosylcobalamin.** Upregulated *Dhc*-genes are enclosed in a solid red box, and transcribed *Dhc* genes are highlighted in dashed red boxes. Transcribed genes from non-*Dhc* organisms are enclosed in a black dashed box.

KB-1 Metagenome Sequence Analysis and Comparative Metagenomics

A metagenome sequencing project for the KB-1 consortium was completed at DoE-JGI, generating 103 Mb of sequence data. The DOE-JGI also sequenced metagenomes from two other *Dhc*-containing enrichment cultures: DonnaII and ANAS. A comparative metagenomic study of these three metagenomes was undertaken to identify common features that are provided by the non-dechlorinating community and are potentially essential to *Dhc* activity. Table 2.2 summarizes key characteristics of the three mixed cultures.

Table 2.2. Characteristics and maintenance conditions for the three *Dhc*-containing enrichment cultures.

	Culture		
Characteristics	**KB-1**	**DonnaII**	**ANAS**
Liquid volume	1.6 L	5.7 L	0.4 L
Stirred	No	Yes	Yes
Temperature	20-22°C	30°C	25-28°C
Electron acceptor	TCE	PCE	TCE
Electron donor	Methanol	Butyrate	Lactate
Feeding frequency	14 days	2 days	4-7 days
Donor loading rate (meeq/L/d)	1.84	4.44	54.5
Acceptor loading rate (meeq/L/d)	0.37	0.44	0.30
Donor eeq/Acceptor eeq	5	10	180
Cobalamin (B_{12}) amended (µg/L)	0.005	1	0.1
Dhc strain(s)	KB-1/PCE & KB-1/VC	*Dhc* 195	ANAS (2 strains)
RDase genes	35	17	17

The metagenomes obtained from the three consortia were automatically annotated using the MG-RAST server (Table 2.3), from which statistically significant differences in community composition and metabolic profiles were determined. Examination of specific metabolic pathways, including corrinoid biosynthesis, methionine biosynthesis, oxygen scavenging, and electron-donor metabolism identified the *Firmicutes*, methanogenic *Archaea*, and the *Delta-Proteobacteria* as key organisms encoding these pathways, and thus potentially producing metabolites *Dhc* requires for growth. A

detailed analysis of the corrinoid biosynthesis pathway suggested a role for methanogens in these communities.

Table 2.3. General features of the metagenome datasets.

Feature	KB-1	DonnaII	ANAS
Type of sequencing	Sanger	454	454 & Sanger
Total number of bases pre-assembly	106,515,530	930,446,714	330,964,688
Number of contigs	6,361	47,030	10,807
Total length of contigs (bp)	14,988,108	24,573,718	30,615,713
Number of singletons	18,629	105,608	15,486
Total length of singletons (bp)	13,487,233	57,708,799	10,450,264
Largest contig (bp)	155,970	121,460	921,258
Average contig size (bp)	2,356	522	2,832
Average G+C content (%)	52.33	52.28	51.91
Protein coding genes	40,766	194,527	60,992
- with COGs	21,857	116,001	39,920
- connected to KEGG pathways	8,077	36,685	11,878
rRNA genes (5S/16S/23S)	18 (7/5/6)	185 (11/62/112)	40 (23/8/9)
tRNA genes	330	818	525
CRISPR count	48	7	57
MG-RAST data			
% *Dhc* in culture*	43.7	31.3	18.2
Metagenome size (bp)*	106,508,248	916,191,214	330,396,345
Average read length*	958	477	547
Number of sequences*	111,162	1,920,396	603,841
Number (%) identified for metabolic analysis[†]	63,352 (57.0)	363,424 (18.9)	222,012 (36.8)
Number (%) identified for phylogenetic analysis[†]	88,888 (80.0)	540,785 (28.2)	294,470 (48.8)

* = post-MG-RAST preprocessing, which removed duplicate reads and nonsense reads from the datasets

†= maximum e-value of 1×10^{-5}, minimum alignment length ~100

This comparative metagenomic analysis identified that similarities across the three consortia are more apparent at the functional level than at the taxonomic level, indicating the non-dechlorinating organisms' identities can vary provided they fill the same function within a consortium. Functional redundancy was identified in each metabolic pathway of interest, with key processes encoded by multiple taxonomic groups. This redundancy likely contributes to the robust growth and dechlorination performance in dechlorinating enrichment cultures (64).

Utility of Microarrays as Site Assessment and Bioremediation Monitoring Tools
Examples for the successful application of microarrays for monitoring gene content and expression in clinical samples, pure microbial and enrichment cultures, and environmental samples exist, but microarrays are "closed" tools meaning that only known genes, for which probes have been designed and incorporated on the array, can be detetected (42, 54, 56, 164, 165). Microarrays are designed to identify genes, for which sequence information is available. The stringency and specificity of microarrays makes them not applicable for biomarker discovery of novel RDases such as *dcpA*, *cfrA* and *dcrA,* since these genes have low similarity to known RDase gene sequences.

A major issue limiting the utility of microarrays (e.g., the RDase gene array) as bioremediation monitoring tools is the requirement for high target gene abundances (i.e., low sensitivity). The RDase array only provided meaningful data using samples with high-target gene abundances (i.e., $>10^4$ target gene copies per mL of groundwater) To achieve such high target gene abundances, biostimulation and bioaugmentation treatment are typically required suggesting that the RDase array may serve as a bioremediation monitoring tool at some bioremediation sites but has limited utility for site assessment prior to the implementation of bioremediation or at most MNA sites. The application (Example 3) presented above demonstrated that the RDase gene array generated false negative results for the known RDase genes, whereas qPCR quantified these RDase genes in the same sample materials. These findings indicate that without target gene amplification (i.e., PCR), the RDase array lacks sensitivity and is prone to generate false negative results. In addition, the array only provided relative target gene abundance

information over a narrow dynamic range (<2 orders of magnitude), and absolute quantitative information cannot be obtained over a broad dynamic range of target gene abundances. At best, relative abundance estimates are possible by comparing samples collected from the same location(s) over temporal scales but a targeted qPCR approach is needed to accurately measure the abundances of the genes of interest. In conclusion, the major shortcomings of the microarray approach when applied to environmental samples include (i) limited sensitivity (i.e., false negative results), (ii) no true quantitative information, and (iii) false positive signals due to cross-hybridization, which makes data interpretation challenging to impossible.

Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE)

The application of native PAGE for identifying and characterizing RDases is a promising approach for assigning specific dechlorination function(s) to RDases and the corresponding genes. BN-PAGE is a protein electrophoresis technique that preserves the native state and activity of proteins. The approach is also applicable to membrane proteins and protein complexes. Native PAGE separation of proteins was combined with proteomics workflows to assign function to RDases and identify the encoding genes (Figure 2.9). These assays were applied to the cDCE- and VC-dechlorinating pure culture *Dhc* strain BAV1, the mixed culture KB-1, the *Dhb*-containing mixed culture ACT-3 (142), the pure culture *Dhgm* strain BL-DC-9, and the mixed cultures KS and RC to identify and characterize the RDases that were expressed during growth with the different substrates.
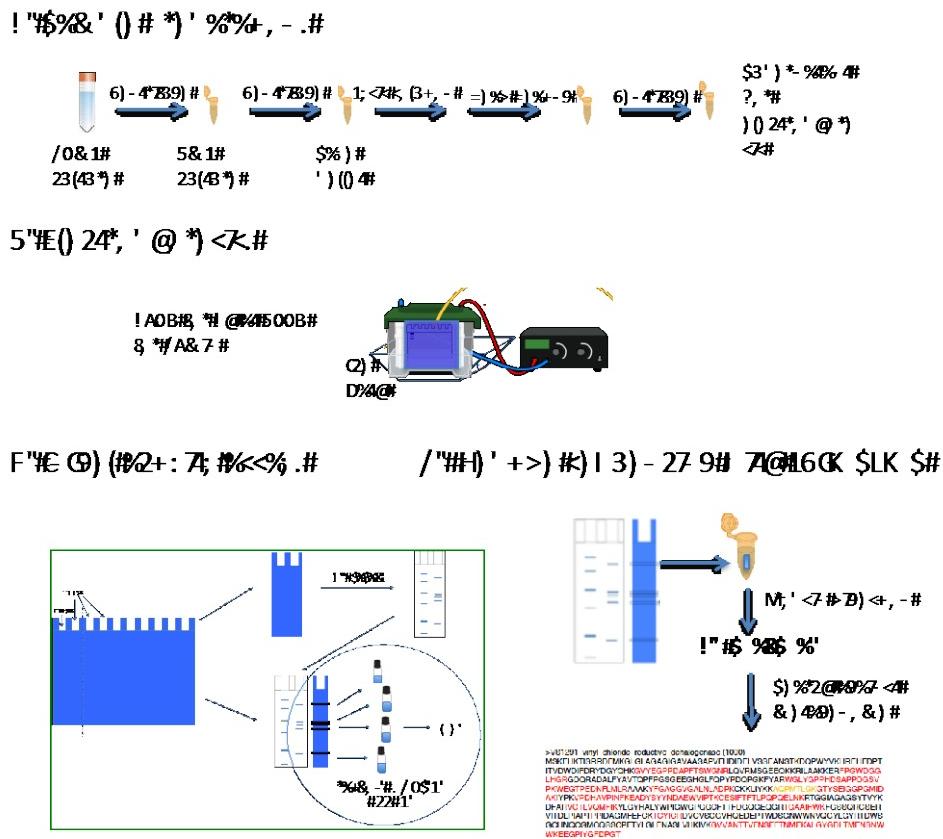
Figure 2.9. Overview of the BN-PAGE procedure. 1) cells are lysed and proteins dissolved with the help of digitonin, a non-ionic detergent; 2) electrophoresis, where each lane (other than MW marker lane) is a replicate of the same sample. Coomassie Blue G is added to the running buffer. 3) RDase assays on gel slices. The first two lanes are cut and stained to locate protein bands. Bands were cut from unstained lanes and assayed. 4) Proteins from gel slices showing dechlorination activity were digested with trypsin and analyzed by LC-MS/MS.

RDase expression was investigated in *Dhc* strain BAV1 cultures and in the KB-1 consortium grown with chlorinated ethenes and 1,2-dichloroethane (141). In cultures of strain BAV1, BvcA was the only RDase detected, revealing that this enzyme catalyzes the dechlorination not only of VC, but also of all DCE isomers and 1,2-dichloroethane, which is in line with the substrate utilization observed for this strain (49). In enzyme assays with cell-free crude extract, BvcA also showed activity towards TCE even though strain BAV1 cannot grow with TCE as electron acceptor (Table 2.4); however, PCE and TCE co-metabolism was observed in strain BAV1 cultures in the presence of cDCE or VC (49). Although the KB-1 metagenome contains 35 different putative RDase genes,

only a small subset of five distinct *Dhc* RDases and one *Geobacter* RDase were
expressed in this mixed culture under all conditions tested (Table 2.4).

Table 2.4. Summary of BN-PAGE analyses for different dechlorinating cultures.

Culture	Culture condition before protein extraction	Chlorinated substrates tested on gel slices	Activity detected?	RDases identified in active gel slices[a]
KB-1 maintained on TCE and methanol	Starved for 5 days then amended with TCE and H$_2$	TCE cDCE tDCE VC 1,2-DCA PCE	Yes Yes Yes Yes Yes Yes	KB1_VcrA (*) KB1_BvcA (*) KB1_GeobRD (*) KB1_RdhA5 KB1_TceA
KB-1 maintained on TCE and methanol (same culture as above)	Starved for 5 days then amended with VC and H$_2$	TCE cDCE tDCE VC 1,2-DCA PCE	Yes Yes Yes Yes Yes Yes	KB1_VcrA (*) KB1_BvcA (*) KB1_GeobRD KB1_RdhA5 KB1_TceA KB1_RdhA1
1,2-DCA KB-1 sub-culture	Grown exclusively on 1,2-DCA and methanol for > 4 years	TCE cDCE tDCE VC 1,2-DCA PCE	Yes Yes Yes Yes Yes Yes	KB1_VcrA (*) KB1_TceA (*) KB1_BvcA KB1_RdhA5
Dhc strain BAV1	Grown on cDCE	TCE cDCE tDCE 1,1-DCE VC 1,2-DCA PCE	Yes Yes Yes Yes Yes Yes No	BvcA
Dhc strain BAV1	Grown on 1,2-DCA	1,2-DCA	Yes	BvcA
Dhgm strain BL-DC-9	Grown on 1,2-DCP	1,2-DCP	Yes	DcpA

[a] The identified RDases are listed in the order of decreasing peptide hit counts.

*The dominant RDases are highlighted with asterisks.

Three of the five expressed RDases included orthologs to the previously identified
chlorinated ethene-dechlorinating enzymes VcrA, BvcA and TceA. KB-1's VcrA
ortholog was the most abundant RDase expressed regardless of growth substrate (VC or
TCE). This study also revealed substrate promiscuity among these three enzymes. If

enzymes have activities toward multiple compounds, this may extend to the activity of intact cells and therefore has implications for their use as specific biomarkers. For example, VcrA is implicated in TCE, cDCE and VC (and possibly 1,2-DCA) dechlorination, not just VC dechlorination. BvcA is implicated in cDCE, tDCE, 1,1-DCE, VC and 1,2-DCA reductive dechlorination. In an attempt to create a nomenclature or at least a framework for understanding the relationship between different RDases that have been, and continue to be discovered, we attempted to reconcile a classification scheme for RDases (65) that was based on function and sequence.

Identification of DcpA Using BN-PAGE and Proteomic Workflows

Cell-free *in* vitro enzyme assays and BN-PAGE demonstrated that *Dhgm* strain BL-DC-9 grown with 1,2-DCP expressed he RDase DcpA. Briefly, crude extracts of strain BL-DC-9 were separated by BN-PAGE, and gel sections were subjected to activity assays (141). The gel section representing the 75-37 kDa gel slice demonstrated 1,2-DCP reductive dechlorinating activity (Figure 2.10). Subsequent SDS-PAGE analysis of the proteins present in the excised gel section showing dechlorinating activity confirmed a major protein band with a molecular mass of 50 kDa, which represents the average molecular weight of known *Dhc* and *Dhgm* RDases. Further analysis using an LC-MS/MS instrument with an LTQ XL mass spectrometer yielded peptides that matched genes on the closed genome of *Dhgm* strain BLDC-9 (NC_014314.1). The analysis demonstrated that the 1,2-DCP RDase was encoded by a gene annotated as putative RDase gene. The gene encoding the 1,2-D RDase was designated *dcpA*, which encodes the 1,2-DCP RDase DcpA. Analysis of the 1,2-DCP-dechlorinating *Dhc* cultures RC and KS (85) indicated that *dcpA* was also present in *Dhc* strains KS and RC. Subsequent transcriptional analysis demostrated that *dcpA* transcription increased when *Dhc* strain RC and KS were grown with 1,2-DCP, thus corroborating that this gene encodes a 1,2-DCP RDase (Figure 2.11).
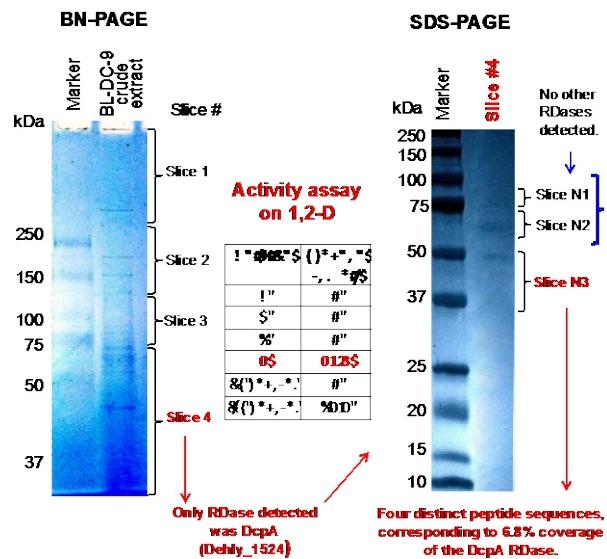
Figure 2.10. RDase gene expression in *Dhgm* strain BL-DC-9 cells grown with 1,2-DCP.
Left panel: BN-PAGE results showing the predominant proteins present and the gel sections that were used in the dechlorinating activity assays. Propene production was confined to slice number 4, which was further separated by SDS-Page (right image). Three gel sections were further analyzed by proteomics and the only RDase detected was DcpA. The marker used was the Precision Plus Protein Standards Kaleidoscope (Bio-Rad, CA). In the activity assays, the positive control consisted of whole cells collected from 1 mL of culture fluid suspended in assay buffer, while the negative control consisted of buffer with no protein added.
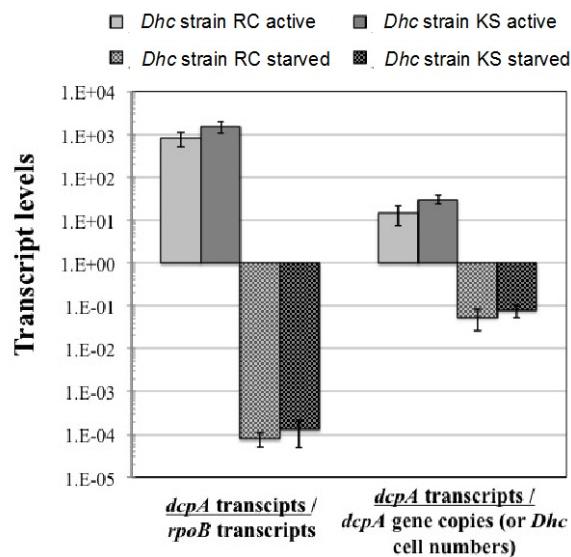
Figure 2.11. Gene expression levels in the 1,2-DCP-dechlorinating cultures RC and KS.
dcpA transcript levels were normalized to *rpoB* or to *dcpA* gene copy numbers. Triplicate qPCR assays were run for each sample and final values represent the average of at least three biological replicate cultures. The error bars depict the standard error.

Figure 2.12 depicts characteristic features of the *dcpA/dcpB* genes in Dhc strain RC and strain KS. Also indicated are the primer binding sites.
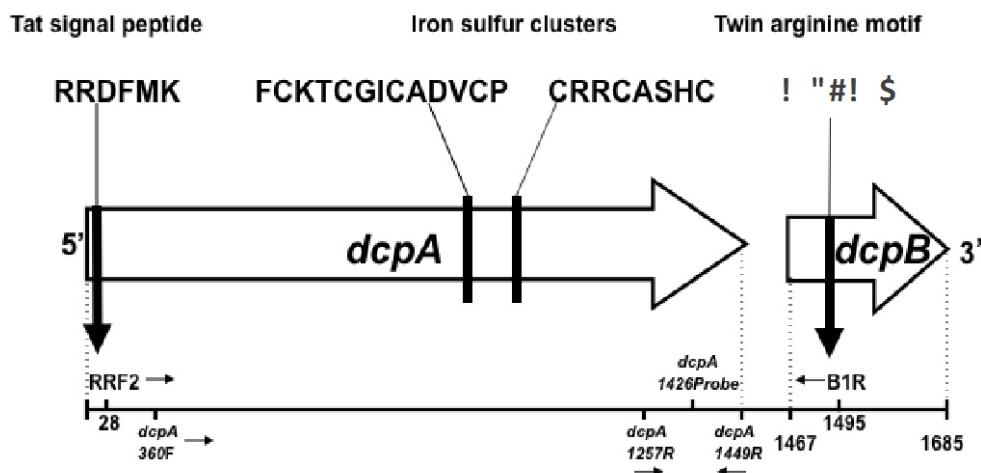
Figure 2.12. Features of the *dcpA/B* genes. The *dcpA* RDases in *Dhc* strains RC and KS share features with other functional RDases: the conserved amino acids for the Tat signal peptide RRXFXK near the N-terminus and two iron sulfurs clusters closer to the C-terminal in the form of FCXXCXXCXXXCP (or FCX2CX2CX3CP) and CXXCXXXC (or CX2CX3C). *dcpB* is located downstream of dcpA and encodes a protein with a conserved motif in the form WYXW. The *dcpA* gene in *Dhgm* also shares these common RDase features. Approximate binding sites for the degenerate primers of putative RDase genes (RRF2 and B1R) as well as *dcpA*-specific primers are indicated.

Identification of CfrA and DcrA Using BN-PAGE and Proteomic Workflows

The BN-PAGE approach was applied to the ACT-3 mixed culture (grown with 1,1,1-TCA) and its two sub-cultures grown with 1,1-DCA or CF. Two novel RDases that were highly similar to each other but catalyzed distinct dechlorination reactions were identified from the *Dhb*-containing mixed cultures. This was accomplished by first assembling the complete genomes of two *Dhb* strains from the metagenomes of the ACT-3 parent culture and the CF-enriched sub-culture (143). Then, BN-PAGE was used to separate crude protein extracts obtained from mixed culture ACT-3 biomass. Gel slices were assayed for dechlorinating activity and associated proteins were identified using LC-MS/MS with the metagenome of the parent culture as the reference database (Figure 2.13). The two RDases identified, designated CfrA and DcrA, shared 95.2% amino acid identity, but

used different substrates: CfrA dechlorinates CF and 1,1,1-TCA, but not 1,1-DCA; DcrA dechlorinates 1,1-DCA, but not CF or 1,1,1-TCA. These two novel RDases share no more than 40% amino acid identity to other confirmed or putative RDases, but both share a twin arginine motif and two iron-sulfur binding motifs found in other RDases. Peptides specific to two putative membrane anchor proteins, designated CfrB and DcrB, were also detected in gel slices (142).
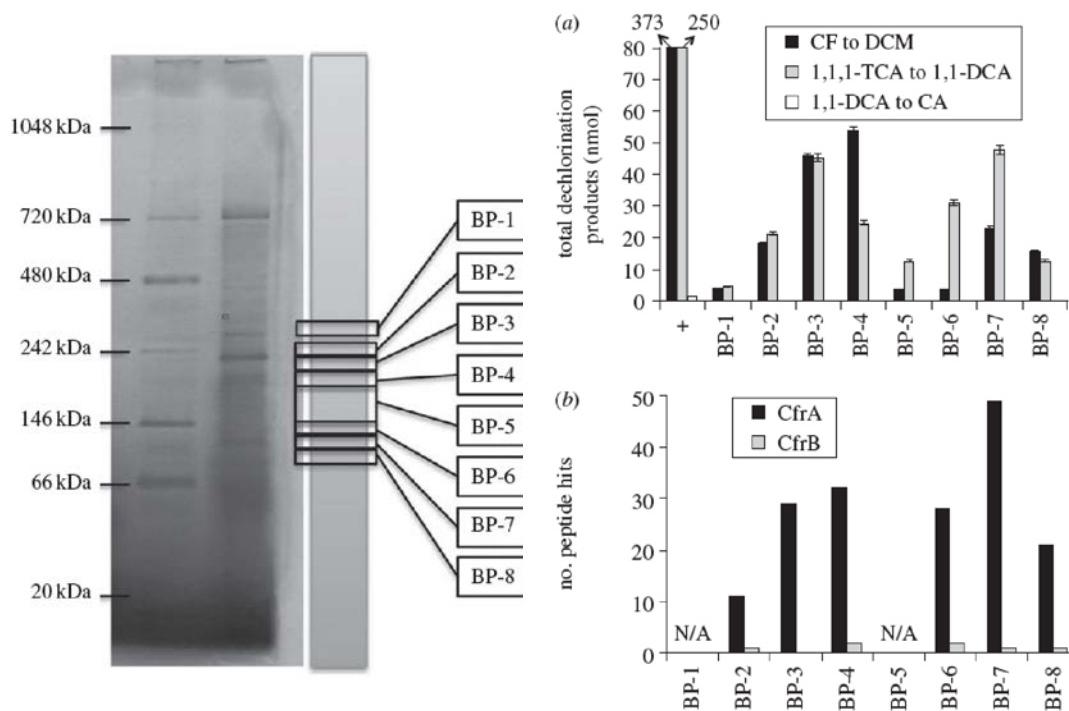
Figure 2.13. Results of BN-PAGE with protein samples from the CF subculture. Left Panel: BN-PAGE gel image showing sliced Band Positions (BP); Right Panel (a) Quantification of dechlorination products in enzyme assays with gel slices. The arrow bars indicate the regular technical error in headspace GC measurements. Plus symbol indicates the positive control, where activity was measured using 20 ml of protein extracts equal to the volume loaded onto each BN-PAGE well. Black bars: DCM detected from CF; grey bars: 1,1-DCA detected from 1,1,1-TCA; white bars: CA detected from 1,1-DCA. Right Panel (b) Counts of LC-MS/MS detected peptide hits in gel slices when searched against curated RdhA and RdhB proteins derived from the ACT-3 metagenome. 'N/A' indicates that the gel slice was not analyzed.

Compound-Specific Stable Isotope Analysis (CSIA)

Although the BioRed project focused on the design of nucleic acid-based biomarkers for monitoring reductive dechlorination of chlorinated solvents, alternate approaches were also explored. CSIA is a promising tool for monitoring *in situ* microbial activity, and enrichment factors (ε values) determined using CSIA can be employed to estimate target compound transformation rates and extents. In collaboration with Dr. Ivonne Nijenhuis and Dr. Hermann Richnow from the Department of Isotope Biogeochemistry at the Helmholtz Centre for Environmental Research in Leipzig, Germany, carbon enrichment factors were measured during the reductive dechlorination of 1,2-DCP to propene. CSIA determined the bulk enrichment factors for 1,2-DCP dichloroelimination in the two distinct *Dhc*-containing cultures RC and KS. The bulk enrichment factors calculated in the two cultures were statistically identical, -10.8 ± 0.9 and -11.3 ± 0.8‰, even though the cultures were derived from geographically distinct locations (126). The consistency of the isotope effects between these cultures indicates that CSIA may be a promising approach to verify and quantify 1,2-DCP dichloroelimination in subsurface environments (38), in particular when combined with quantitative monitoring of the *dcpA* gene implicated in 1,2-DCP-to-propene reductive dechlorination.

ε values for *Dhc*-catalyzed dechlorination reactions in the PCE-to-ethene reductive dechlorination pathway have been reported (58, 68, 69, 109, 113, 148). What had not been accomplished was to determine the reproducibility between independent experiments, variability between different *Dhc* strains, and congruency of ε values measured in *Dhc* pure cultures and *Dhc*-containing mixed cultures. In experiments conducted with pure cultures of *Dhc* strain BAV1, ε values for 1,1-DCE, cDCE, tDCE, and VC were -5.1, -14.9, -20.8, and -23.2‰, respectively. The ε value for 1,1-DCE dechlorination was 48.9% higher than the value reported in a previous study (81), but ε values for other chlorinated ethenes were equal between independent experiments. For the dechlorination of cDCE and VC by *Dhc* strains BAV1, FL2, GT, and VS, average ε values were -18.4 and -23.2‰, respectively. cDCE and VC ε values determined in pure *Dhc* cultures with different RDase genes (e.g., *vcrA* or *bvcA*) varied by less than 36.8 and 8.3%, respectively. In consortium BDI, ε values for cDCE and VC dechlorination were -

25.3‰ and -19.9‰, or 31.6% higher and 15.3% lower, respectively, compared to the average ε value for *Dhc* pure cultures. As cDCE and VC ε values are all within the same order-of-magnitude and fractionation is always measured during reductive dechlorination catalyzed by *Dhc*, these findings support CSIA application as a complementary approach for monitoring *in situ* cDCE and VC reductive dechlorination (38).

To date, there has been little information about CSIA applicability for chlorinated methanes. Moreover, published enrichment factors (ε) observed during the biotic and abiotic degradation of chlorinated alkanes, such as CT, 1,1,1-TCA and 1,1-DCA, range from −26.5‰ to −1.8‰ and illustrate a system where similar C–Cl bonds are cleaved but significantly different isotope enrichment factors are observed. Biotic degradation of CF to DCM was carried out by the *Dhb*-containing culture DHB-CF/MEL, also shown to degrade 1,1,1-TCA and 1,1-DCA. The carbon isotope enrichment factor (ε) measured during biodegradation of CF was −27.5‰ ± 0.9‰, consistent with the theoretical maximum kinetic isotope effect for C–Cl bond cleavage. Unlike 1,1,1-TCA and 1,1-DCA, reductive dechlorination of CF by the *Dhb*-containing culture shows no evidence of suppression of the intrinsic maximum kinetic isotope effect (Table 2.5). Such a large fractionation effect, comparable to those published for cDCE and VC suggests CSIA can identify and monitor biodegradation of CF, as well as fingerprint natural versus anthropogenic sources of CF in soils and groundwater (20).

Table 2.5. Measured ε values for CF and 1,1,1-TCA reductive dechlorination in mixed culture ACT-3.

Compound	Condition	ε value
CF	Biotic (ACT-3)	-27.5
1,1,1-TCA	Biotic (ACT-3)	-1.8 to -1.5
CT[a]	Abiotic	-26.1 to -26.5
1,1,1-TCA[b]	Abiotic	-15.8 to -13.6

[a]   Values obtained for experiments of abiotic reductive cleavage of CT by Fe (II)/goethite and Fe(II) porphyrin (32).

[b]   Values obtained for experiments of abiotic reductive cleavage of 1,1,1-TCA by Cr(II), Fe^0, and Cu and Fe mixtures (70).

Task 3: Design and Validate qPCR Assays for the Most Promising Biomarker Genes and their Transcripts

The rapid and sensitive evaluation of environmental samples and contaminated site materials for microbial parameters is crucial for science-based decision-making and for fully exploiting the potential of the microbiology to achieve pollutant detoxification. qPCR has emerged as a robust technology to enumerate DNA sequences (i.e., genes) or RNA following reverse transcription (RT) of transcripts into complementary DNA (cDNA). qPCR technology has been broadly applied in the medical field for years, and general qPCR guidelines have been established (18); however, the extension of the approach to undefined environmental samples is not trivial. For example, groundwater may contain compounds (e.g., inhibitors) that interfere with target gene amplification or contain non-target DNA sequences that are amplified during PCR. Such interferences can cause false negative and false positive results, and careful qPCR assay design and evaluation that considers the challenges associated with environmental samples is pivotal.

Two commonly employed qPCR approaches utilize TaqMan or SYBR Green fluorogenic reporter chemistries for quantifying genes and transcripts (19). Both approaches are used to answer questions regarding specific gene or gene transcript abundances in dechlorinating cultures, microcosms and environmental samples (8, 47, 49, 122, 139, 157). In addition to a forward and a reverse primer targeting the gene of interest, TaqMan assays utilize a specific linear hybridization probe that has a 5' fluorescent reporter and a 3' quencher molecule. As the polymerase synthesizes a double stranded DNA molecule, the probe molecule is degraded by the 5'-to-3' exonuclease function of the Taq polymerase, separating the reporter from the quencher thus allowing measurable fluorescent light emission (59). In contrast, SYBR Green assays take advantage of the SYBR Green fluorescent dye that specifically binds to double stranded nucleic acids (dsDNA). Because the dye binds all dsDNA, both specific and non-specific amplicons or even primer dimers produce fluorescence signals. SYBR Green- and TaqMan-based assays generally produce comparable qPCR data when defined laboratory cultures are used; however, the SYBR Green assays may result in false positive amplification or overestimation of the true target gene abundance(s) when DNA is obtained from

environmental samples (48). Because of the potential ambiguities that can arise from SYBR Green-based detection chemistry, it is recommended to transition qPCR assays to the more robust and more sensitive TaqMan approach when environmental samples (e.g., groundwater) are analyzed.

To date (January 2013), 44 different bacterial species are known to contain at least one (putative) RDase gene. A 16S rRNA gene-based phylogenetic tree with the known genera comprising at least one species harboring a (putative) RDase gene is shown in Figure 3.1 (67). This phylogenetic tree highlights the known bacterial diversity that encompasses the ability to catalyze the reductive dehalogenation of a wide range of chlorinated compounds. Since the realization that *Dhc*, which are members of the *Dehalococcoidia* (87), were responsible for the detoxification of DCEs and VC, much research was directed at enumerating members and genes belonging to this genus; however, as the initial steps in the reductive dechlorination of chlorinated ethenes rely on other bacterial genera including *Dhb*, *Desulfuromonas*, *Desulfitobacterium,* and *Geobacter* (60, 61, 138, 140). Consequently, a comprehensive suite of qPCR assays for assessing the reductive dechlorination of chlorinated ethenes must include biomarkers for these other dehalogenating genera in addition to *Dhc* biomarkers.

Knowledge of dechlorinator phylogeny implicated in specific reductive dechlorination reactions offers opportunities to design 16S rRNA gene-targeted qPCR assays that provide valuable information about the microorganisms contributing to contaminant degradation at contaminated sites. The detoxification of chlorinated ethenes has been linked to the presence of *Dhc* (57) and three relevant RDase genes associated with this process have been identified (76, 86, 97, 111). Dechlorination of TCE to cDCE and VC has been linked to the *tceA* gene (73, 97). More importantly, the *bvcA* and *vcrA* genes encode RDases involved in the reduction of cDCE and VC to nontoxic ethene, and consequently the abundance of these two RDase genes, together with the abundance of the *Dhc* 16S rRNA gene, correlate with the complete dechlorination of chlorinated ethenes (76, 111, 122).
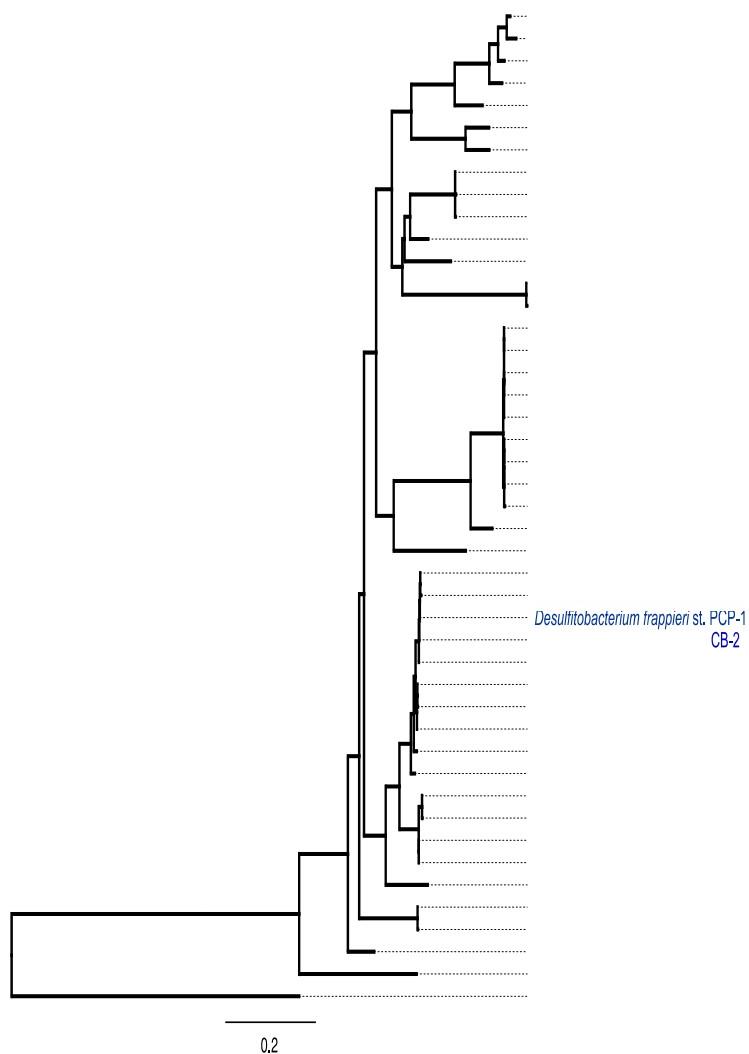
Figure 3.1. Maximum likelihood phylogeny of the 16S rRNA gene sequences from known organisms containing at least one (putative) RDase gene. Bootstrap support values are based on 100 bootstrap bipartitions. For organisms with multiple 16S rRNA genes, one representative sequence was chosen.

As the *Dhc* 16S rRNA gene and the three RDase genes *tceA*, *bvcA* and *vcrA* emerged as excellent targets for assessing the potential for complete reductive dechlorination at sites impacted with chlorinated ethenes, a large number of RDase genes were identified on *Dhc* genomes and in metagenomes of *Dhc*-containing consortia (66, 78, 80, 105, 133). Additional RDase genes were identified from the genomes of other organhalide-respiring bacteria and through efforts to amplify RDase genes directly from environmental samples

(23, 62, 76). Of the 255 putative RDase genes included in Figure 3.2, only 13 have a characterized function. In addition to the previously discussed *tceA*, *vcrA*, and *bvcA* genes, other relevant RDase genes include *pceA* responsible for PCE to TCE and PCE to cDCE dechlorination (29, 120, 122), *dcaA, cfrA and dcrA* involved in reductive dechlorination of chlorinated ethanes (143), *mbrA* implicated in TCE reductive dechlorination to *trans*-DCE (22), *cbrA* contributing to the reductive dechlorination of chlorinated benzenes (2), and *dcpA* encoding a 1,2-DCP RDase.

1 - TceA (*Dehalococcoides ethenogenes* st. 195, TCE)
2 - BvcA (*Dehalococcoides* st. BAV1, VC)
3 - VcrA (*Dehalococcoides* st. VS, VC)
4 - MbrA (*Dehalococcoides* st. MB, 1,1,2,2-TeCA)
5 - CbrA (*Dehalococcoides* st. CBDB1, chlorobenzenes)
6 - PceA (*Dehalococcoides ethenogenes* st. 195, PCE)
7 - PceA (*Desulfitobacterium hafniense* Y51, PCE)
8 - CprA (*Desulfitobacterium hafniense* st. PCP-1, chlorophenols)
9 - CprA (*Desulfitobacterium dehalogenans*, chlorophenols)
10 - PrdA (*Desulfitobacterium* sp. KBC1, PCE)
11 - CfrA (*Dehalobacter* sp. ACT-3, 1,1,2-TCA, 1,1,1-TCA, CF)
12 - DcrA (*Dehalobacter* sp. ACT-3, 1,1,2-TCA, 1,1-DCA)
13 - DcaA (*Dehalobacter* sp. WL, 1,2-DCA)
14 - DcrA (*Desulfitobacterium dichloroeliminans*, 1,2-DCA)

pceA

Other chloroethene RDases

Other known substrates

Figure 3.2. Maximum likelihood tree of 255 confirmed and putative RDase genes.
Bootstrap support values are based on 100 bootstrap bipartitions. Organism names are colored approximately by phyla, as in Figure 3.1 (65)

DNA Extraction and qPCR Analysis

A prerequisite for applying regular PCR, qPCR or RT-qPCR is the extraction and purification of nucleic acids (i.e., DNA, RNA) from the environmental sample material. A wide variety of DNA extraction and purification methods have been developed for different sample matrices (95, 96, 106). Specific protocols taylored towards the sample material of interest generally outperform commercial kits in terms of nucleic acid yield and purity (106); however, the benefits provided by commercial kits, such as the Powersoil Kit (MO BIO), allow for consistent DNA extraction from a range of environmental samples, including sediment materials and groundwater biomass collected onto membrane filters (124). To date, there is no standard method, by which total microbial nucleic acids are obtained from sediments, soils or groundwater samples, and extraction efficiencies vary greatly (25, 112). Ideally, a readily available commercial nucleic acid kit has an extraction efficiency of >90%, recovers DNA in high qualtity in terms of fragment length and purity, and removes any inhibitory compounds that could interfere with downstream analyses. Unfortunatley, there is a tradeoff between ease of use, effort, sample throughput, extraction efficiency, and quality of the nucleic acid preparation, and no single kit will provide uniform results with different sample materials.

A few commonly applied methods to extract nucleic acids from laboratory cultures and environmental samples were compared and optimized (123). For pure and enrichment cultures, the Qiagen DNA Tissue Kit provided high quality DNA that could be used for a majority of the nucleic acid-based analyses; however, this protocol does not incorporate steps for removing inhibitory compounds (e.g., humics, metals) that may be present in environmental samples. The MO BIO PowerSoil Kit removes inhibitory compounds and was found to yield consistent and reproducible DNA preparations from biomass collected from groundwater. Although a compromise, this method combines efficiency, nucleic acid recovery and quality, and removal of inhibitors with the flexibility to accommodate a variety of environmental samples materials including groundwater and aquifer solids. Of course, a variety of different nucleic acid extraction methodologies exist and many will provide PCR-amplifyable DNA. Most important for site monitoring is that the same

methodology is applied over temporal and spatial scales, so that the data collected over time and from different locations (wells) at a site can be directly compared (124, 125).

Primer and Probe Design

Primers and probes (Table 3.1) were designed to specifically detect and quantify organism-specific (i.e., 16S rRNA gene-targeted assays) and process-specific (e.g., RDase gene-targeted assays) biomarker genes described in Task 2. Figure 3.3 illustrates some of the degradation pathways that have been identified for a subset of chlorinated solvent groundwater contaminants. In addition to the pathways, Figure 3.3 depicts the known bacterial genera involved (16S rRNA gene-targeted assays) and several RDase genes that have been implicated in different dechlorination steps.
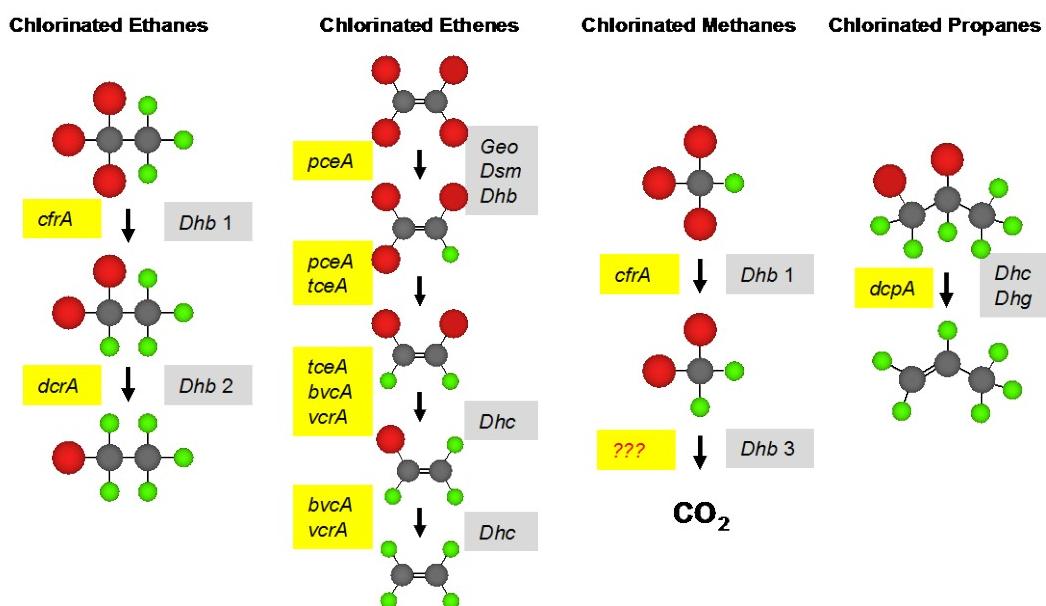
Figure 3.3. Biomarkers involved in the reductive dechlorination of chlorinated ethenes, chlorinated ethanes, chlorinated methanes and chlorinated propanes. The yellow boxes indicate the availability of the process-specific (i.e., RDase gene-targeted) biomarker gene qPCR assays. The grey boxes show bacteria, for which organism-specific (i.e., 16S rRNA gene-targeted) qPCR assays are available. Process-specific biomarker genes for DCM degradation have not been identified.

To achieve standardized procedures and expedite analysis, TaqMan probes and primer pairs were designed to have the same melting temperatures (Tm) and reaction conditions as standard TaqMan *Dhc* 16S rRNA gene qPCR assays and available RDase gene qPCR

assays (Tm of 60˚C for the primers and 70˚C for the probe). For each gene target, primers and probes were designed using NCBI's GenBank primer design tool to identify the regions useful for primer design while the Primer Express 3.0 software (Applied Biosystems) was used to refine the choice of primers and probes within the specific regions (122).

Table 3.1. Key organisms and genes involved in the reductive dechlorination process. Summary of the current knowledge of organism-specific (i.e., 16S rRNA genes) and pathway-specific (i.e., functional) biomarker genes directly (RDase gene) or indirectly (corrinoid scavenging, hydrogenases) involved in in the transformation of chlorinated solvents.

Target Group	Gene	Dechlorination biomarker	Organism-specific assay		Assay	Reference
			Genus[a]	Species		
Bacteria	16S rRNA	Phylogenetic			☐TM	(122)
Dhc sp.	16S rRNA	Phylogenetic	☐		☐TM	(50)
Dhc	*pceA*	PCE/TCE → cDCE	☐		-[b]	unpublished
Dhc	*tceA*	TCE → VC	☐		☐TM	(122)
Dhc	*vcrA*	cDCE → ethene	☐		☐TM	(122)
Dhc	*bvcA*	cDCE → ethene	☐		☐TM	(122)
Dhc	*mbrA*	TCE → tDCE	☐		-[b]	unpublished
Dhc	*dcpA*	1,2-DCP → propene	☐		☐TM	unpublished
Dhc (CV)	*fdhA*	Formate dehydrogenase - function unknown		☐	☐SYBR	unpublished
Dhc strain (P) BAV1	*fdhA*	as above		☐	☐SYBR	unpublished
Dhc strain (P) BAV1	*hupF*	NiFe hydrogenase		☐	☐SYBR	unpublished
Dhc (P)	*vhuG*	Cytoplasmic [Fe] hydrogenase		☐	☐SYBR	unpublished
Dhc (P)	*cobU*	Cobinamide kinase		☐	☐TM	unpublished
Dhc (P)	*cbiZ*	Adenosylcobinamide amidohydrolase		☐	☐TM	unpublished
Dhgm sp.	16S rRNA	Phylogenetic	☐		☐SYBR ☐TM	(160) unpublished
Dhgm sp.	*dcpA*	1,2-DCP → propene	☐		☐TM	unpublished

Target Group	Gene	Dechlorination biomarker	Organism-specific assay		Assay	Reference
			Genus[a]	Species		
"Dehalobium chlorocoercia" DF-1	16S rRNA	Phylogenetic	☐		-[b]	unpublished
Dhb restrictus	16S rRNA	Phylogenetic		☐	☐SYBR ☐TM	(47) unpublished
Dhb sp. RM	16S rRNA	Phylogenetic		☐	☐TM	(75)
Dhb sp.	*pceA*	PCE/TCE → *c*DCE	☐		-[b]	unpublished
Dhb sp.	*cfrA*	1,1,1-TCA → 1,1-DCA	☐		☐SYBR -[b]	(143)
Dhb sp. CF	*cfrA*	CF → DCM	☐		☐SYBR -[b]	(143)
Dhb sp. DCA	*dcrA*	1,1-DCA → CA	☐		☐SYBR -[b]	(143)
Dhb sp.	*rdh1*	1,2-DCA → ethene	☐		☐SYBR	(46)
Desulfitobacterium spp.	*pceA*	PCE/TCE → *c*DCE	☐		☐TM	unpublished
Desulfuromonas michiganensis	*pceA*	PCE/TCE → *c*DCE		☐	☐TM	unpublished
Geobacter lovleyi KB-1	16S rRNA		☐		☐SYBR	(29)
Geobacter lovleyi SZ	16S rRNA	Phylogenetic		☐	☐SYBR ☐TM	(10)
Geobacter lovleyi SZ	*pceA*	PCE/TCE → cDCE			☐SYBR ☐TM	unpublished
Geobacter lovleyi KB-1	*pceA*	PCE/TCE → cDCE		☐	☐SYBR	unpublished
Geobacter lovleyi SZ	*pceA*	PCE/TCE → cDCE		☐	☐SYBR ☐TM	unpublished
Sulfurospirillum sp. KB-1	16S rRNA	Phylogenetic	☐		☐SYBR	(29)
Sulfurospirillum sp. KB-1	*pceA*	PCE/TCE → *c*DCE	☐		☐SYBR -[b]	(29) unpublished

☐   specificity for genus or species

[a] Genus-specific assays are available; however, not all members of the genus are capable of catalyzing the respective dechlorination step

☐SYBR   assay available for SYBR Green

☐TM   assay available for TaqMan or TaqMan-MGB

[b] A specific assay is in the testing phase

The pipeline for developing each qPCR assay followed standard operating procedures (SOPs) to ensure that all assays exhibited acceptable quality control standards (18). After suitable primer/probe combinations were identified, the primers were tested using SYBR Green assays with dilutions of pure culture genomic DNA or plasmid DNA containing the gene of interest. Primers that did not amplify the target gene with 90-110% efficiency were discarded and new primers were designed and tested to meet this criterion. Melting curve analysis determined whether single symmetric peaks were obtained (indicative of specifc target gene amplification).

Each target gene was then amplified from genomic DNA using primers external to the qPCR primer-amplified fragment, and the larger fragment (or near-complete gene) was ligated into the pCR2.1 cloning vector (Invitrogen) with both ampicillin and kanamycin antibiotic resistance markers. The plasmids carrying the target gene fragments were propagated in *E. coli* for ease of use and to archive individual clones for generating positive control DNA for qPCR assays. All *E. coli* clones were as glycerol freezer stocks stored at -80˚C. Plasmid DNA was extracted from *E. coli* clones grown in ampicillin or kanamycin (100 µg/mL) containing lysogeny broth (LB) (118) using the QiaPrep plasmid isolation kit. Gel electrophoresis and ethidium bromide staining verified a high plasmid yield and the absence of genomic DNA. If necessary, the plasmid band was excised from the gel and purified from any contaminating genomic DNA before plasmid DNA was quantified using an UV/Vis spectrophotometer or the NanoDrop (Thermo Scientific). To validate spectrophotometric DNA quantification, the plasmid DNA concentration of select clones was verified by fluorometry using the Picogreen dye (Invitrogen) and comparison with a DNA standard curve using the 96-well plate BioTek plate reader. Following plasmid quantification, 10-fold dilution series beginning at 1 ng/µL were prepared as described (122). These 10-fold plasmid DNA dilutions were used to generate qPCR standard curves to quantify gene targets in DNA samples obtained from laboratory cultures and environmental samples.

One example for the development of a qPCR assay for a target gene within the *Chloroflexi* was demonstrated using cultures of *Dhc* strain KS and strain RC, both capable of dechlorinating 1,2-DCP to propene via a dichloroelimination reaction (85, 126). The *dcpA* gene encoding a 1,2-DCP RDase was identified in Task 2. Figure 3.4 shows quality control experiments that were incorporated into primer and probe design for the *dcpA* gene assay using the SYBR Green detection chemistry and melting curve analysis.
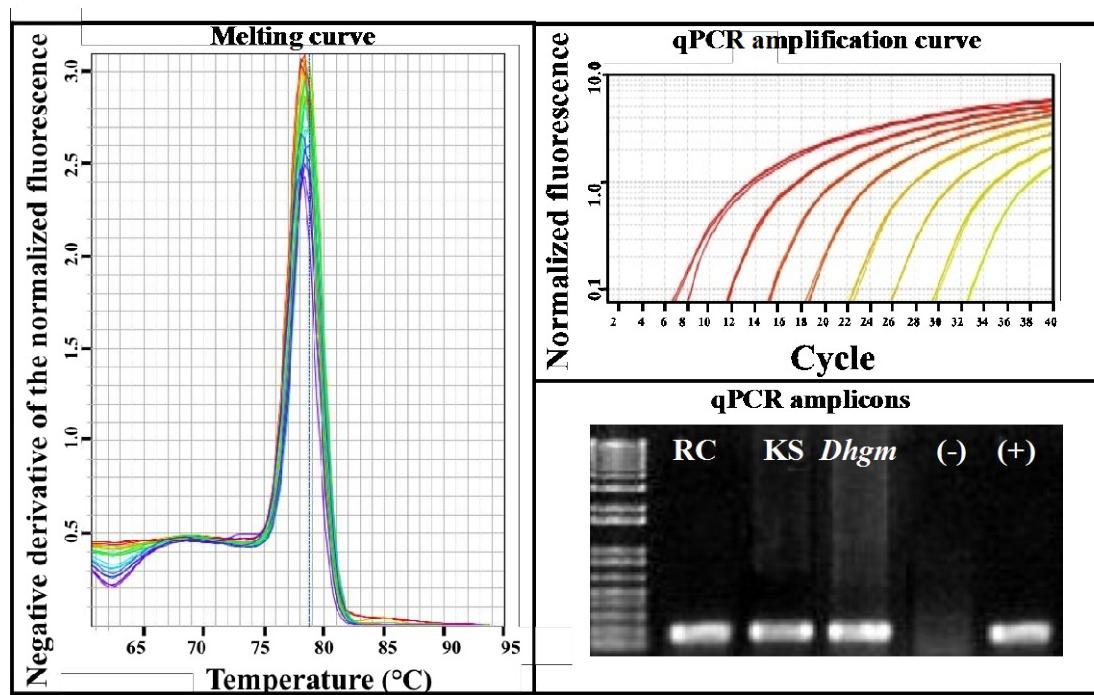
Figure 3.4. SYBR Green-based qPCR assay experiments targeting the *dcpA* gene. The left panel shows the melting curve analyses performed with the SYBR Green assay usind *dcpA*-targeted primers and genomic DNA of *Dhc* culture RC and culture KS and *Dhgm* strain BL-DC-9 as templates. Shown in the top-right panel are examples of TaqMan amplification curves obtained with the *dcpA* gene-targeted primers and the *dcpA* TaqMan probe for a 10-fold dilution series of template DNA spanning a range of 10^8 to 10^2 *dcpA* copies per reaction. Additionally, *dcpA* TaqMan PCR amplification products were visualized using gel electrophoresis to confirm assay specificity (bottom-right panel). Lanes 1-3: 10 ng of template DNA of *Dhc* culture RC, *Dhc* culture KS, and *Dhgm* strain BL-DC-9, respectively. Lane 4 is a no template control and lane 5 corresponds to a reaction that had 2 ng of plasmid DNA carrying a single copy of a *dcpA* gene fragment. The left lane on the gel shows the 1 kb Plus DNA Ladder (Invitrogen).

After confirming that the primers generated a standard curve with an amplification efficiency of 90-110% in the SYBR Green assay, triplicate standard curves were prepared with the TaqMan probe using a plasmid standard generated with the *dcpA* gene of *Dhc* strain KS (Figure 3.5).
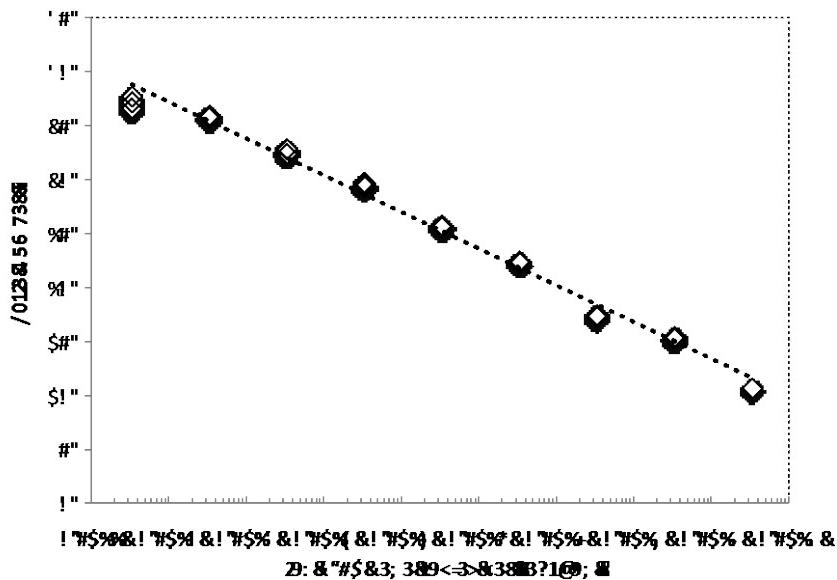
Figure 3.5. A TaqMan-based standard curve for the *dcpA* qPCR assay using 10-fold serial dilutions of plasmid DNA with a single copy of a *dcpA* gene fragment. The TaqMan-based qPCR assays were performed using plasmid DNA carrying the *dcpA* gene fragment of *Dhc* strain KS (gray squares). The standard curve shown has a dynamic range of 10^8 to 10^0 gene copies per mL of template DNA.

After validating the qPCR assay with the TaqMan probe, the assay's specificity was evaluated using available DNA templates from laboratory cultures and environmental samples that dechlorinated/failed to dechlorinate 1,2-DCP. A prerequisite of all qPCR assays was that no signals were obtained with template DNA from cultures that did not contain the *dcpA* gene, as well as DNA obtained from enrichment cultures and microcosms that did not show 1,2-DCP reductive dechlorination activity. An example of the application of the *dcpA* qPCR assay is shown for Third Creek sediment samples during stages of enrichment following consecutive transfers (Figure 3.6). The qPCR assays demonstrate the increase in *Dhc* 16S rRNA gene and *dcpA* gene abundances following enrichment with 1,2-DCP as electron acceptor.

	Dhc RC	Dhc KS
■ Dhc 16S rRNA gene copies	6.1E+07	5.2E+07
□ dcpA gene copies	6.6E+07	6.2E+07
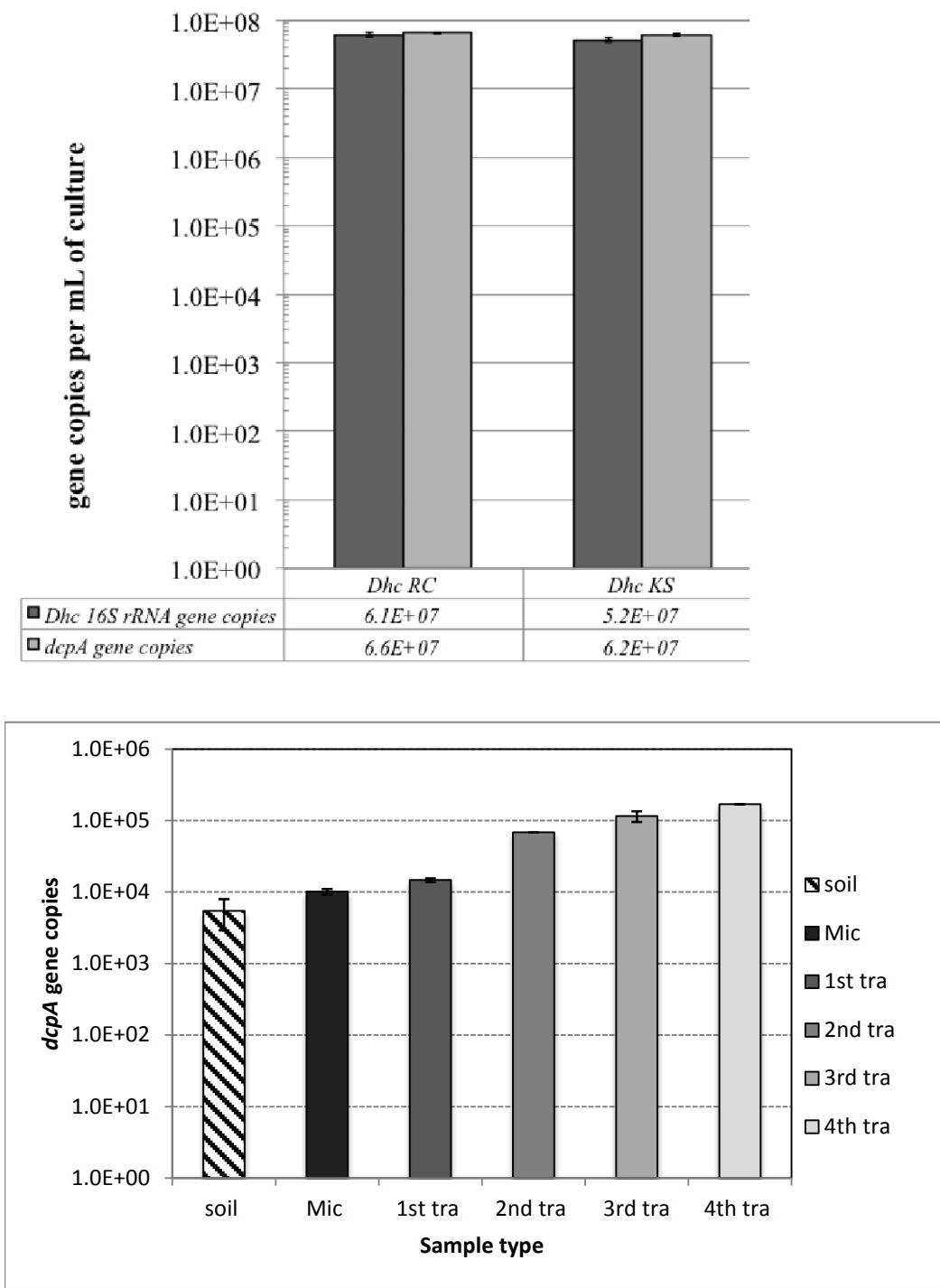
Figure 3.6. Application of the TaqMan-based qPCR assay for the *Dhc* 16S rRNA gene and the *dcpA* gene in Third Creek sediments and dechlorinating enrichment cultures.
Top panel: Average *dcpA* copies per mL of culture (RC and KS enrichments); also indicated are the average 16S rRNA gene copies determined with qPCR.
Bottom panel: Average *dcpA* copies per g of Third Creek sediment (bar on left labeled "soil") and average *dcpA* copies per mL in microcosms (mic) and transfer cultures (tra). Error bars indicate the standard deviations of triplicate qPCR reactions and duplicate DNA extractions.

Accuracy of qPCR Assays

In order to ensure that the qPCR analysis reflected actual cell numbers, *Dhc* cells in strain BAV1 pure cultures were quantified using direct cell enumeration and indirect (DNA-based) approaches. Acridine Orange and SYBR Gold counts were obtained for direct enumeration. SYBR Gold staining was performed by M. Dumas and E. Wommack, University of Delaware. DNA was extracted using the Qiagen Tissue Kit with reported modifications (122) and the bacterial and *Dhc* 16S rRNA genes, as well as the strain BAV1 *bvcA* gene were enumerated with qPCR. Both the 16S rRNA gene and the *bvcA* gene occur as single copy genes on the strain BAV1 genome and their abundances equal the *Dhc* cell numbers (122).

The results displayed in Figure 3.7 demonstrate that qPCR slightly underestimates the actual cell numbers relative to the direct count methods. Most likely, the slightly lower cell numbers obtained with qPCR enumeration reflect target gene loss during the DNA extraction process. Nevertheless, the qPCR data for the bacterial and *Dhc* 16S rRNA gene and *bvcA* gene abundances and the direct counts were within 2-3 fold of each other.
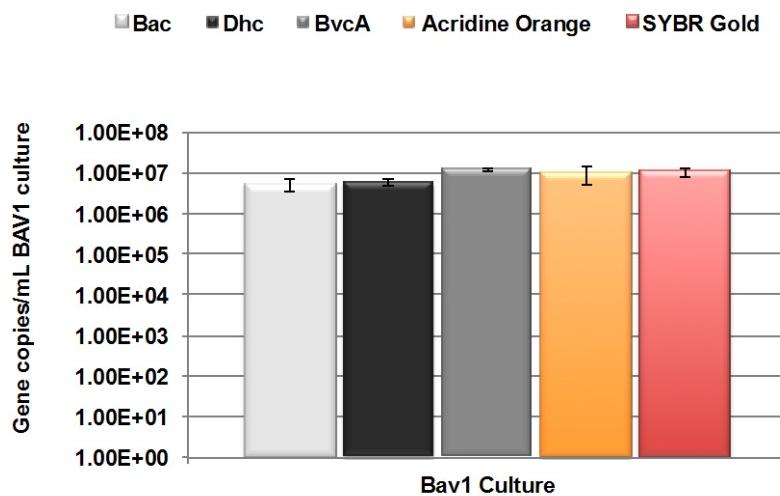
Figure 3.7. Comparison of qPCR quantification of *Dhc mccartyi* strain BAV1 to epifluorescence microscopic cell counts. *Dhc* strain BAV1 cells were enumerated with two independent direct cell count methods using epifluorescence microscopy following Acridine Orange and SYBR Gold staining. TaqMan qPCR used general bacterial and *Dhc* 16S rRNA gene-targeted primer pairs, as well as a *bvcA*-targeted primer pair.

Singleplex versus Multiplex qPCR

After the selection of relevant target genes and validation in singleplex qPCR assays, the potential for simultaneously quantifying two targets in the same PCR tube (i.e., multiplex PCR) was evaluated. Multiplex approaches require careful primer and probe design and additional optimization but use fewer expensive chemicals and allow for higher sample throughput so that the cost per sample can be reduced. Determining the choices and combinations of fluorophores for the TaqMan probes required in multiplex applications is a trial and error process, and the effectiveness of a multiplex assay may even depend on which fluorophore is combined with which probe.

Cross reactivity of probes with the incorrect target in a multiplex assay can lead to spurious qPCR results. If increased probe specificity is necessary for the development of a successful multiplex assay, TaqMan 3'-minor groove-binder (MGB) probes can be used (145). TaqMan MGB probes are short probes (as short as possible without being shorter than 13 nucleotides in length) that contain a 3' chemical modification that modulates their interaction with the target sequence. MGB probes form more stable DNA hybrids with single-stranded target DNA sequences, which result in higher melting temperatures (Tm) of the probes (4, 79). Because of the shorter probe length compared to TaqMan probes without the MGB, the greater difference in Tm for matched and mismatched probes allows for increased probe specificity and discrimination between DNA sequences with as little as a single basepair substitution (79, 101, 163).

To explore the utility of multiplex approaches for enumerating *Dhc* in environmental samples, multiplex qPCR assays were designed to quantify the *Dhc* 16S rRNA gene and another target gene: (i) a modified *Dhc** 16S rRNA gene fragment and (ii) the firefly luciferase gene *luc*. In the first strategy, a single primer set that amplified both target genes ensured equal amplification efficiencies (132). The *Dhc** 16S rRNA gene fragment had a 4 base pair (bp) consecutive modification in the sequence targeted by the TaqMan probe. Hence, two different TaqMan MGB probes discriminated between the wildtype *Dhc* 16S rRNA gene and the modified *Dhc** 16S rRNA gene fragment. In addition, the *luc* gene was used in multiplex qPCR assays to evaluate enumeration of

both target genes in multiplex assays using different amplification primers and TaqMan probes. In singleplex reactions, no significant differences in amplification efficiency or detection limit were observed for the *Dhc* 16S rRNA gene or either the *Dhc** 16S rRNA gene fragment or the *luc* gene, allowing quantification over greater than 6-orders of magnitude (Hatt et al., manuscript in review). The amplification efficiencies were not significantly different ($p < 0.05$) in simplex assays of *Dhc* 16S rRNA (6-FAM) and the *Dhc** 16S rRNA gene fragment (JOE) or between *Dhc* and the *luc* gene. No inhibition of amplification was observed in multiplex assays containing equal target gene abundances (Table 3.2). A more detailed description of the design and application of an internal amplification control is available in the report prepared for SERDP project ER-1561.

Table 3.2. ANOVA results comparing the slopes of regression lines of the standard curves for simplex and multiplex qPCR targeting the *Dhc* 16S rRNA gene, the *Dhc* * 16S rRNA gene, and the *luc* gene. [a]

Target	Slope	Y-int	R^2	Slope	Y-int	R^2	df	F-ratio	p
***Dhc** 16S**									
Simplex *Dhc*	-3.61	40.66	0.997						
Simplex *Dhc** 16S	-3.45	38.78	1.000						
Multiplex *Dhc* vs *Dhc** 16S	-3.64	40.62	1.000	-3.56	39.08	0.995	1.40	2.46	0.13
Simplex vs Multiplex *Dhc*	-3.61	40.66	0.997	-3.64	40.62	1.000	1.52	0.07	0.79
Simplex vs Multiplex *Dhc** 16S	-3.45	38.78	1.000	-3.56	39.08	0.995	1.34	0.68	0.42
Luciferase gene (*luc*)									
Simplex *Dhc*	-3.55	39.37	0.999						
Simplex *luc*	-3.45	38.54	1.000						
Multiplex *Dhc* vs *luc*	-3.44	38.72	0.998	-3.33	37.96	0.999	1,60	3.65	0.06
Simplex vs Multiple *Dhc*	-3.55	39.37	0.999	-3.44	38.72	0.999	1,50	2.21	0.14
Simplex vs Multiplex *luc*	-3.45	38.54	1.000	-3.33	37.96	0.999	1,47	2.57	0.12

[a] Regression lines were drawn using the means of at least three independent 10-fold serial dilution series of plasmid DNA containing each gene target.

To more thoroughly evaluate the multiplex qPCR assays, the *Dhc* 16S rRNA gene and the *Dhc** 16S rRNA gene fragment target DNAs were mixed in different ratios. The assay amplified the two targets linearly over 7 orders of magnitude when both target DNA concentrations were within a 10-fold difference. When the two target genes were mixed in opposing unequal quantities (with 10-fold dilutions of one target starting at <10 copies and ranging to 10^8 gene copies and the other starting at 10^8 gene copies and decreasing to <10 copies), only the target gene that was present in greater than or equal abundance to the opposing target gene was detected and quantified Figure 3.8.
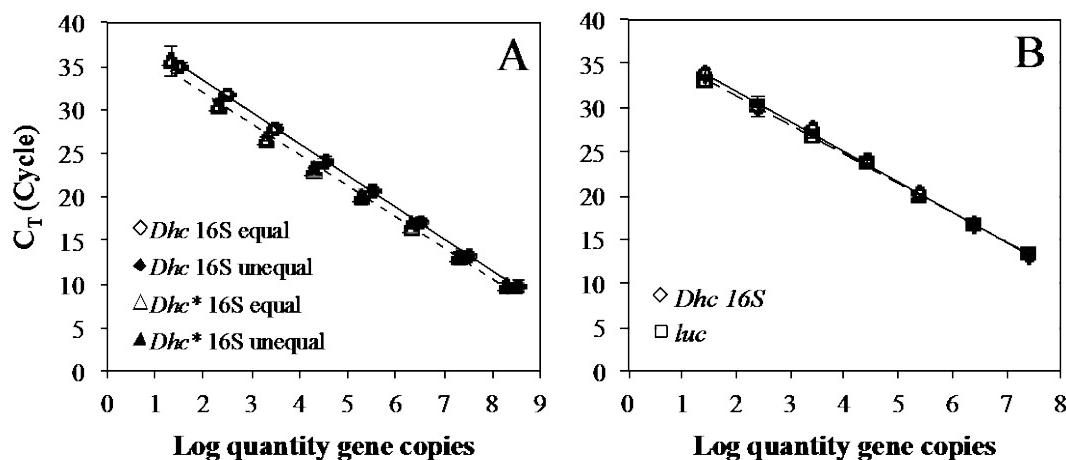
Figure 3.8. Standard curves for the *Dhc* 16S rRNA gene (diamond) and the *Dhc** 16S rRNA gene (triangle) and *luc* gene (square). (A) Standard curves for the *Dhc* 16S rRNA gene and the *Dhc** 16S rRNA gene targets (triangles) carried on the respective plasmids, respectively, assayed in multiplex qPCR format. Standard curves were generated with a mixture of either equal concentrations of the two plasmids ranging from ~10^8 to 10 plasmids/assay (open symbols) or with a mixture of opposing or unequal plasmid concentrations (i.e., ~10^8 to 10 and ~ 10 to 10^8 plasmids/assay) (solid symbols). The mean values for at least three independently obtained standard curves of each type are shown, and error bars represent the standard deviation. Open symbols for both targets extend for the entire range but are obscured by the filled symbols at the higher plasmid concentrations. (B) Comparison of standard curves for *Dhc* 16S rRNA gene and luc targets (circles) assayed in multiplex qPCR assays. The standard curves are based on the mean values of five independently prepared dilutions using the plasmid carrying each target. Error bars represent the standard deviation.

As demonstrated by the comparison of multiplex to singleplex assays in opposing gradients and different target gene abundances, the use of the multiplex approach is challenging. First, the intensity of different fluorophores may bias quantifcation and very careful (i.e., laborious) qPCR testing is required. Another potential issue is the cross reactivity of primers and/or probes with target and/or non-specific DNA, what diminishes the amplification efficiency and target gene quantification thresholds. A major issue is the need for both target genes to be present within 1-2 orders of magnitude in abundance for optimal primer and probe reaction with both DNA templates. Because the target gene copy numbers are not known *a priori* in environmental samples, the multiplex approach will have limited utility for most environmental samples (e.g., for bioremediation monitoring).

qPCR Targeting Transcripts of Functional Genes (i.e., mRNA)

The qPCR approach is easily expanded to monitor gene expression and quantify target gene transcripts (i.e., reverse transcriptase [RT]-qPCR). Following total RNA extraction, the sample is DNase treated to remove any remaining DNA. The RNA is reverse transcribed into complementary DNA (cDNA), and quantified as well as evaluated for quality using the Agilent 2100 BioAnalyzer (Agilent Technologies). The development and optimization of RNA extraction protocols is described in Task 4. Critical for a meaningful results interpretation is the initial incorporation of an internal standard prior to RNA extraction to quantify biomarker transcript loss during the extraction and reverse transcription process in addition to the thorough removal of contaminating DNA during sample preparation (123) (see also Task 4 below).

Task 4: Explore if GeneTranscription-to-Gene Abundance Correlation Factors Estimate *In Situ* Reductive Dechlorination Activity

The goal of this effort was to explore if biomarker gene expression correlates with dechlorination rates. If successful, biomarker transcript-to-gene abundance ratios could be correlated with reductive dechlorination rates measured under defined conditions in the laboratory. Subsequently, the measurement of transcript-to-gene abundance ratios in groundwater samples would be determined to evaluate the utility of these ratios for estimating or predicting *in situ* dechlorination rates at contaminated sites.

RNA Extraction

To establish activity correlation factors that are useful for estimating reductive dechlorination rates, two key requirements must be met. First, accurate quantification of transcripts (mRNA) must be achieved, and second, the abundance of functional (i.e., RDase) gene transcripts must correlate with the respective RDase activity. Efficient RNA extraction procedures with complete DNA removal are pivotal for meaningful quantification, data analysis and interpretation. Extraction methods that are not optimized to recover a representative RNA pool can lead to underestimation of transcript of interest abundance or even produce false negative results. To account for extraction losses, a known amount of commercially available luciferase mRNA can be added as an internal standard (72, 115, 137). In this approach, the commercial control RNA (1 µg/µL) is diluted 1:10 to obtain a 100 ng/µL working stock solution with 1×10^{11} luciferase transcripts per µL. The luciferase mRNA is added directly to the biomass prior to the cell lysis step. Based on the fractional recovery of the luciferase standard mRNA as cDNA, *Dhc* biomarker RNA recoveries generally vary between 10-30% (9). RNA recovery is calculated by dividing the total number of luciferase cDNA molecules enumerated with qPCR by the total number of luciferase transcripts added to the sample (123). It should be noted that the luciferase mRNA standard approach does not recognize and quantify RNA loss due to inefficient cell lysis. Nevertheless, the luciferase mRNA approach improves RT-qPCR accuracy.

Cell density, the type of organism and stage of growth all impact the yield and quality of RNA obtained from any particular sample (114). Therefore, in order to collect sufficient biomass for RNA analysis from groundwater and dilute laboratory cultures, RNA extractions procedures were modified. Groundwater samples were filtered onto a membrane (e.g., MO BIO water filters, Durapore hydrophilic membrane filters, Millipore Sterivex cartridges) prior to extraction (123, 124). Using this method, less time was spent in cell harvesting (about 5 min compared to 10-30 min for biomass collection by centrifugation) thus minimizing the risk of RNA degradation, and bigger volumes could be easily managed.

RNA recoveries have traditionally been normalized to the transcript abundance of a housekeeping gene such as *rpoB*; however, housekeeping gene expression is not constant under all growth conditions (147). Additionally, in the case of *Dhc*, the current *rpoB* gene-targeted primers do not distinguish between *Dhc* strains with different RDase genes (41). Therefore, with the best possible quantification approaches available, we evaluated whether transcript-to-gene abundance ratios of genes involved in reductive dehalogenation by *Dhc* and other organisms could be correlated to dechlorination activity.

RNA Stability and Longevity
RNA is less stable than DNA representing a challenge for analysis of transcripts in environmental samples and for determining accurate correlations with gene abundances. In order to preserve the less stable RNA transcripts for downstream applications, a number of commercially available RNA preservatives were tested (e.g., RNAprotect® Bacteria Reagent from Qiagen, RNA*later* (Ambion/Life Technologies) to determine which RNA stabilizing agent provided the best protection and highest transcript recovery. To compare the effectiveness of the RNA stabilizing reagents, biomass was collected by centrifugation from 1 mL of a fumarate-grown *Anaeromyxobacter dehalogenans* culture. The biomass was immediately frozen with or without an RNA stabilizing agent (e.g., RNAprotect® Bacteria Reagent) and stored at -80°C until extraction. Pellets were thoroughly suspended in 0.5-1 mL of the stabilizing solution. All samples were stored at

4°C overnight prior to freezing in order to allow the preservative to fully permeate the cells (123). RNA was extracted from three technical replicates using the Qiagen RNeasy kit. From the comparison of different RNA preservatives used, the RNA Bacterial Protect (Qiagen) yielded the best results in terms of RNA quality (Figure 4.1A) and quantity (Figure 4.1B). RNA is a labile biomolecule and good laboratory practice is pivotal for protecting RNA from hydrolysis by RNA-degrading enzymes (RNases) and maintaining RNA integrity (17, 40, 129, 134, 156). All plastic materials used were RNase-free and all surfaces and equipment were treated with an RNase inactivating solution (RNaseZap®, RNase *AWAY®*, etc.). Figure 4.1 demonstrates the effects of different preservation methods on RNA integrity and the qPCR results for select reductive dechlorination biomarker genes (i.e., the *Anaeromyxobacter dehalogenans* 16S rRNA gene transcripts). Samples were visualized with the BioAnalyzer to verify RNA integrity and quality, and qPCR was used to enumerate 16S rRNA molecules.
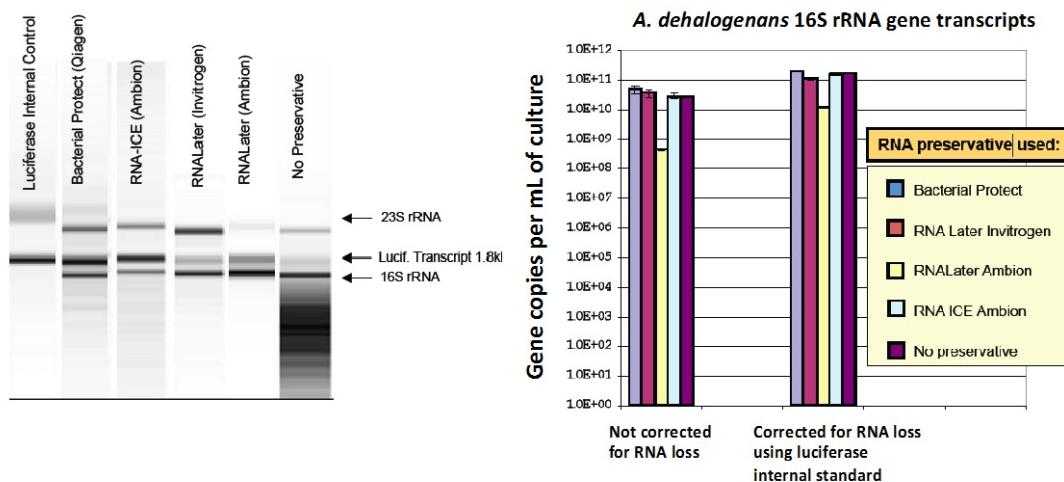
Figure 4.1. Optimizing one RNA extraction method while applying different preservation solutions. The effects of different RNA preservatives were tested to boost RNA quantity and quality. Transcripts were quantified using RT-qPCR. Luciferase mRNA served as an internal control. For these experiments, triplicate RNA extractions were performed using *Anaeromyxobacter dehalogenans* biomass grown with acetate and fumarate. The RNA preservatives RNAprotect bacteria Reagent (Qiagen), RNAlater (Invitrogen), RNAlater (Ambion), and RNAlater-ICE (Ambion) were compared to a no preservative control.

Reductive dechlorination biomarker transcripts can be measured readily in actively dechlorinating cultures using RT-qPCR. As shown in Figure 2.11, gene expression levels of the *dcpA* gene in 1,2-DCP-dechlorinating cultures RC and KS demonstrated that transcript levels could be normalized to either the *rpoB* or to *dcpA* gene copy numbers. The RT-qPCR results showed upregulation of *dcpA* gene transcription in actively dechlorinating RC and KS cultures as compared to cultures that had consumed all 1,2-DCP. When *dcpA* transcripts were normalized to *rpoB* genes, a greater difference was noted in gene upregulation between active and starved cells, compared to *dcpA* transcripts normalized to 16S rRNA gene transcripts.

The RT-qPCR approach was applied to groundwater samples from a TCE-contaminated site near Milledgeville, GA, USA, to explore if the RNA extraction and quantification procedures developed in the laboratory were effective in quantifying transcripts from environmental samples. Further, we explored if the effect of holding times on the RT-qPCR results and analyzed groundwater samples shipped on ice immediately upon arrival in the laboratory, and replicate groundwater containers were analyzed following an 8-day storage at room temperature (22°C). Samples were also collected for DNA analysis to normalize transcript copies to gene copies in the groundwater samples. Groundwater samples from two wells (MW-7 and RW-2) with different VOC concentrations were obtained and microbial biomass was collected from 200-500 mL of groundwater by vacuum filtration onto a 0.22 µm pore size, 47 mm diameter, polyethersulfone membrane filtration unit (MO BIO Ultraclean Water DNA Isolation Kit, MO BIO Laboratories Inc., Carlsbad, CA). RT-qPCR of well MW-7 samples analyzed immediately upon arrival or after 8 days of storage at room temperature (22°C) showed no significant difference ($t_{0.05}$, 4) for any of the transcripts analyzed (total bacterial 16S rRNA, *Dhc* 16S rRNA, and *bvcA*, *vcrA* and *tceA* mRNA). In contrast, significant differences in intranscript abundances ($t_{0.05}$, 4) were observed between RW-2 groundwater samples that were analyzed immediately upon arrival and after 8 days of storage (Figure 4.2). *Dhc* 16S rRNA and *bvcA*, *vcrA* and *tceA* transcripts decreased by about one order of magnitude in samples from well RW-2 following groundwater storage at room temperature. These results indicated a varied response of samples exposed to the same treatment (i.e., 8 days

of storage at room temperature). While a decrease in transcript abundance following storage was not unexpected, the significant differences between groundwater samples from different wells in terms of transcript stability were surprising. The incubation changed significantly the transcript profile of reductive dechlorination biomarkers in samples from well RW-2 when compared to the same samples processed immediately. This was not true for the samples analyzed from well MW-7, which did not reveal any significant change in the biomarker transcript profiles. The concentration of chlorinated ethenes in MW-7 was lower than in RW-2, which may indicate an influence of the metabolic activity of the microorganisms at the time of sampling at the transcriptional level in response to a shift in the environmental conditions.
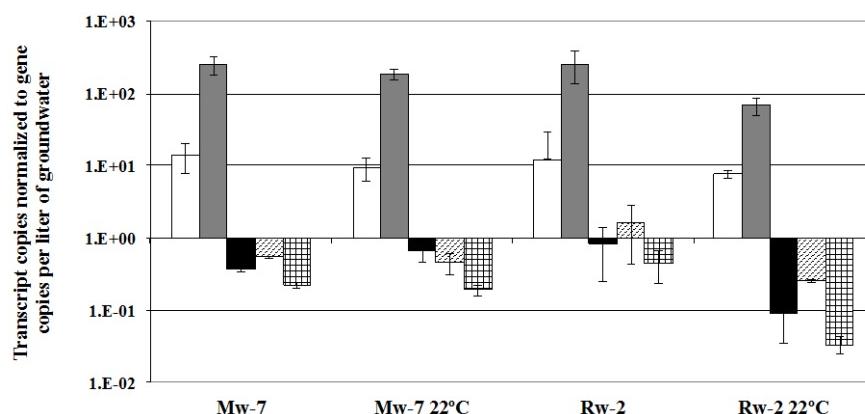
Figure 4.2. Relative gene-transcript quantification in groundwater samples. Replicate samples were processed immediately and following an 8-day incubation period at room temperature. Biomarker transcript copies were normalized to gene copies, and are represented by bars and grouped by well and treatment; total bacterial 16S rRNA molecules (white), *Dhc* 16S rRNA molecules (gray), *bvcA* transcript abundance (black), *vcrA* transcript abundance (diagonal stripes) and *tceA* transcript abundance (tiles). Data labeled with 22°C represent transcript copies for samples incubated at 22°C for 8 days after arrival, while the other samples are transcript copies in the samples processed upon arrival. Each sample represents average values of triplicate samplings of each well. Error bars are ± SD.

In order to correlate transcript abundance to activity, it is imperative that a sample's transcription profile accurately reflects the transcriptome at the time of sample collection. Rifampicin (Rif) is an inhibitor of transcription initiation (136) and can be added to

bacterial cultures to prevent new transcript biosynthesis. Rif has not been used to halt

transcription in *Dhc*-containing cultures or in environmental samples. To investigate the

effect of Rif on transcription in *Dhc* containing cultures, VC-fed BDI cultures were

grown until VC was depleted, at which point the cultures were amended with TCE or

TCE plus Rif. Rif was added at a concentration of 200 μg per mL of culture fluid. The

effect of Rif on TCE-fed BDI cultures is shown in Figure 4.3. Since all *Dhc* strains

present in the BDI culture harbor a single copy of the 16S rRNA gene and one copy of

either the *bvcA*, *tceA*, or *vcrA* RDase gene, normalization of gene transcripts to gene

copies represent the transcripts per cell.
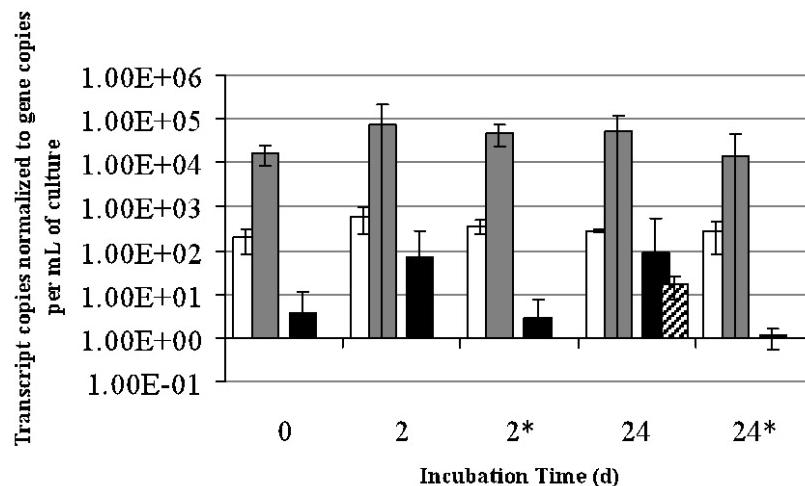
Figure 4.3. Effect of rifampicin on biomarker transcription profiles after substrate addition to the dechlorinating consortium BDI. Triplicate VC-depleted BDI cultures were provided with either TCE or TCE plus Rif. Asterisk (*) indicates that the sample received Rif. The cultures were sampled on Day 0 and one day after TCE and Rif addition (Day 2), and 3 weeks after TCE and Rif addition (Day 24). cDNA and gDNA samples were analyzed by qPCR. The bars bars show transcripts abundances per cell of bacterial 16S rRNA molecules (white), *Dhc* 16S rRNA molecules (gray), *bvcA* transcripts (tiles), *vcrA* transcripts (black) and *tceA* transcripts (diagonal stripes). Each sample represents average values of three cultures. Error bars are ± SD.

Five mL of each culture were sampled for RNA extraction before the addition of TCE

(Day 0) and 48 hours after the addition of TCE or TCE plus Rif (Day 2). At each

sampling event, 1 mL of each culture was collected for DNA analysis for normalization

of transcript copies to gene copies. Cultures amended with Rif ceased dechlorination

activity. After 3 weeks (Day 24), an additional 5 mL of each culture were sampled for

RNA extraction. Initially, VC-depleted cultures exhibited low levels of *vcrA* transcripts. After the addition of TCE (Day 2), *vcrA* transcription was up-regulated. The addition of Rif prevented the up-regulation of *vcrA* transcription and after 48 hours, the transcript levels equaled those observed in VC-depleted cultures before the addition of TCE plus Rif. After 24 days of incubation with TCE, both *vcrA* and *tceA* transcript levels increased in cultures without Rif. In cultures containing Rif, the levels of *vcrA* transcripts decreased slightly but *vcrA* mRNA remained quantifiable. These findings indicate that Rif effectively halts transcript biosynthesis in *Dhc* and that the transcription profile in cultures with Rif remained unchanged, suggesting that Rif addition at the time of sampling may be a suitable approach for providing the most accurate snapshot of the transcriptome at the time of sampling.

Correlating Transcript to Gene Ratios with Reductive Dechlorination Activity

Generally, the number of transcripts within a cell serves as a measure of gene activity and also as an indicator of the activity of the corresponding enzyme system. According to textbook microbiology, transcripts are produced in response to specific stimuli (e.g., the presence of a substrate) and are rapidly degraded when the substrate has been consumed. This concept has been developed for model organisms like *E. coli* and appears to apply broadly, at least to organisms that share basic features with *E. coli*. To establish activity correlation factors that are useful for estimating reductive dechlorination rates, two key requirements had to be met. First, accurate quantification of transcripts (mRNA) must be achieved, and second, the abundance of functional (i.e., RDase) gene transcripts must correlate with the respective RDase activity. Efficient RNA extraction and reverse transcriptase protocols were established (see above) and the inclusion of an internal standard approach enabled accurate transcript quantification. To evaluate if transcript abundances correlated with reductive dechlorination activity, a semi-batch cultivation vessel was designed (Figure 4.4).

Consortium BDI was provided with TCE as the electron acceptor, and fed lactate, which was readily fermented to acetate, propionate and hydrogen. To prevent oxygen leakage into the reactor and provide sufficient hydrogen, the required electron donor for *Dhc*, hydrogen was supplied at a pressure of 1 psi to the cultivation vessel. This culture served as a source of "synchronized" biomass comprising cells with assumed uniform transcriptomes (i.e., active *Dhc* cells were in a similar state of growth/activity). Ideally, a continuous culture vessel (i.e., chemostat) would be used to ensure that all cells of the *Dhc* strain(s) of interest would be in a similar state of growth and the same sets of genes are active (i.e., have synchronized transcriptomes); however, sustaining *Dhc* activity and growth in a chemostat where the dilution rate equals the growth rate has been challenging due to the extensive maintenance efforts. True chemostat cultivation was impractical for performing extended experiments, and the semi-batch incubation vessels shown in Figure 4.6 were used. These vessles were easy to maintain and monitor, and yielded biomass suitable for nucleic acid extraction and the quantification of biomarker genes and transcripts. A series of semi-batch culture experiments were initiated and monitored for dechlorination activity as well as biomarker gene and transcript abundances. Initial results indicated that RDase gene transcription correlated with dechlorination activity and the presence of chlorinated ethene(s) present in the batch cultures. These findings suggested that measurable mRNA turnover occurred in cells maintained under laboratory conditions, which was consistent with other studies (73, 83, 119, 120, 121, 128)
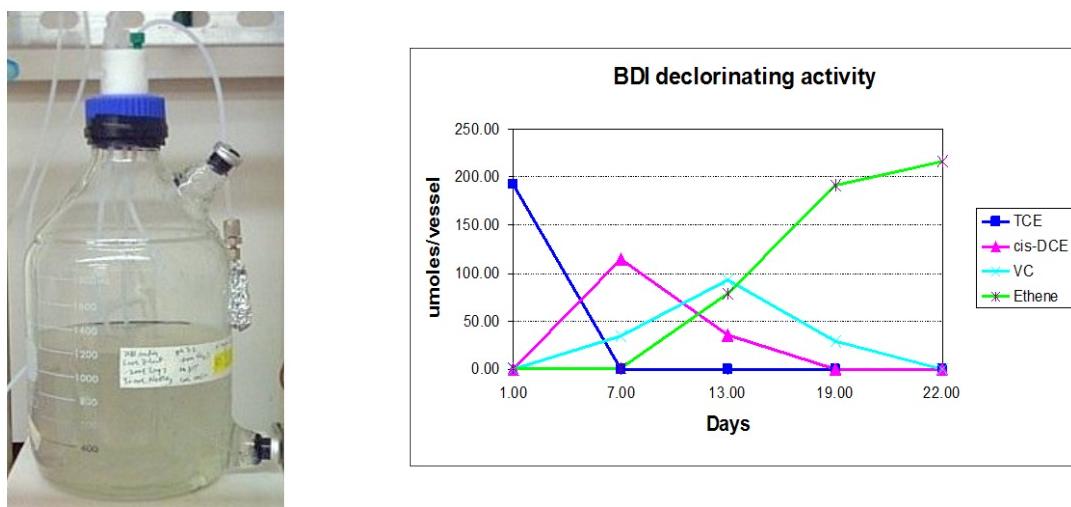
Figure 4.4. Semi-batch culture system for maintenance of consortium BDI. The culture was maintained with TCE as electron acceptor. Culture suspension (200 mL) was removed and replaced with fresh medium weekly. The medium pH was maintained between pH 7 and 7.2, and lactate and hydrogen were provided as electron donors.

To date, very little information about transcript turnover in *Dhc* has been collected. Obviously, rapid turnover (i.e., degradation of mRNA) following substrate (e.g., VC) consumption is a prerequisite for correlating VC dechlorination activity with biomarker transcript abundance per cell. To address this issue, reductive dechlorination biomarker genes and transcripts were measured in the *Dhc*-containing consortium BDI during active dechlorination and during extended phases of starvation (i.e., no chlorinated electron acceptor available). The experiments with consortium BDI suggested that the turnover of at least some *Dhc* RDase transcripts is slow with half-lifes in the order of weeks. Even in cultures starved for extensive periods (many months), some RDase biomarker transcripts could be readily quantified (Figure 4.5). This phenomenon has been observed with other *Dhc* transcripts, and there is some evidence from the KB-1 whole genome arrays, that many transcripts may last for weeks to years.

Obviously, the longevity of mRNA is relevant for meaningful data interpretation. The data depicted in Figure 4.5 demonstrate that as substrates (i.e., TCE and VC) were depleted, copies of the biomarker gene transcripts (i.e., *tceA* and *vcrA* mRNA) decreased

but did not disappear, and were detected at constant concentrations for long periods of time under starvation (i.e., no chlorinated electron acceptor provided).

Another factor complicating the correlation between transcripts and reductive dechlorination activity is the lack of knowledge of the regulatory network that controls RDase gene transcription. Ideally, the chlorinated electron acceptor acts as a specific inducer of gene activity whereas other, unspecific stimuli have no effect on RDase gene activity. Experiments with the BDI consortium revealed that stress conditions affected RDase gene transcription, and RDase gene transcripts were produced and/or maintained even though no reductive dechlorination activity occurred.
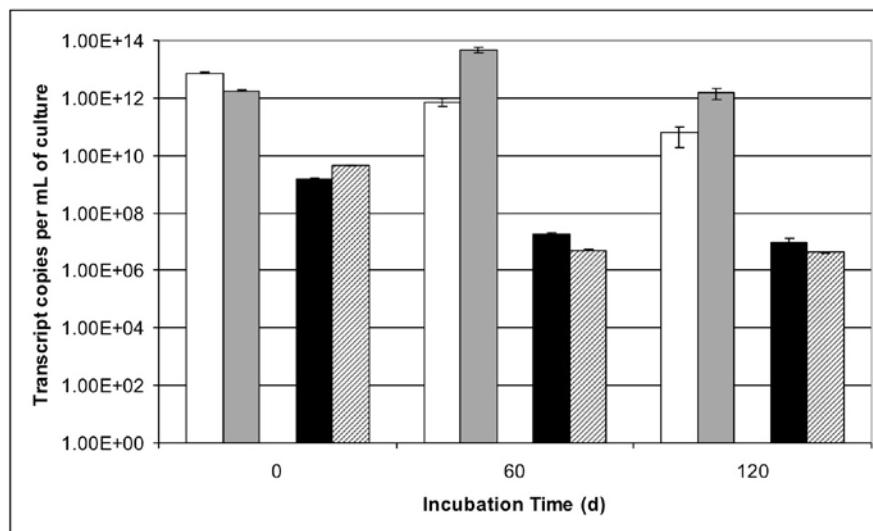
Figure 4.5. Transcript abundance monitored over time in starved laboratory dechlorinating mixed cultures. Data are represented in bars for total bacterial 16S rRNA (white), *Dhc* 16S rRNA (gray) and the RDase gene transcripts *bvcA* (tiles), *vcrA* (black) and *tceA* (diagonal stripes). Each sample represents average values of triplicate samplings of each time point. Error bars show the standard deviation.

Batch culture experiments demonstrated that exposure of the BDI consortium to air (i.e., oxygen) or temperatures above 45°C inhibited all dechlorination activity (9, 37). Following oxygen removal, TCE-to-VC dechlorination recovered but VC-to-ethene

dechlorination did not, suggesting that the *Dhc* strains catalyzing the VC-to-ethene dechlorination step are more susceptible to oxygen inhibition (Figure 4.6). qPCR analysis detected a 1-1.5 order-of-magnitude decrease in the number of *Dhc* biomarker genes (i.e., the *Dhc* 16S rRNA gene and the RDase genes *tceA*, *vcrA*, and *bvcA*) in the oxygen-amended cultures, but these biomarkers remained quantifiable in oxygen-inhibited, non-dechlorinating cultures. RT-qPCR detected *Dhc* 16S rRNA, *tceA* and *vcrA* transcripts in the oxygen-amended, non-dechlorinating cultures (Figure 4.7). Hence, qPCR analysis of biomarker genes failed to distinguish viable, dechlorinating cells from irreversibly inhibited (non-viable) *Dhc* cells.
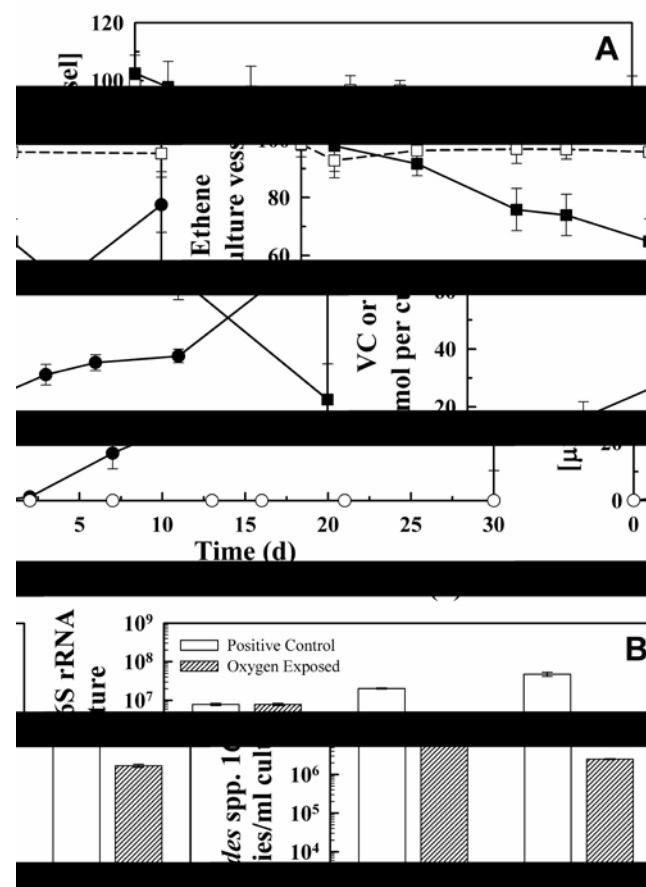
Figure 4.6. Dechlorination performance and qPCR analysis of the VC-fed BDI consortium. (A) Dechlorination performance of the VC-fed BDI consortium in the oxygen-amended (open symbols, dashed lines) or positive control (closed symbols, solid lines) cultures. Symbols: dark squares; VC; dark circles; ethene. (B) qPCR analysis of *Dhc* 16S rRNA gene copy numbers in the oxygen-amended and positive control cultures. All data points represent average values from triplicate cultures, and error bars represent one standard deviation. When error bars are not visible, they are smaller than and therefore hidden behind the data symbols.
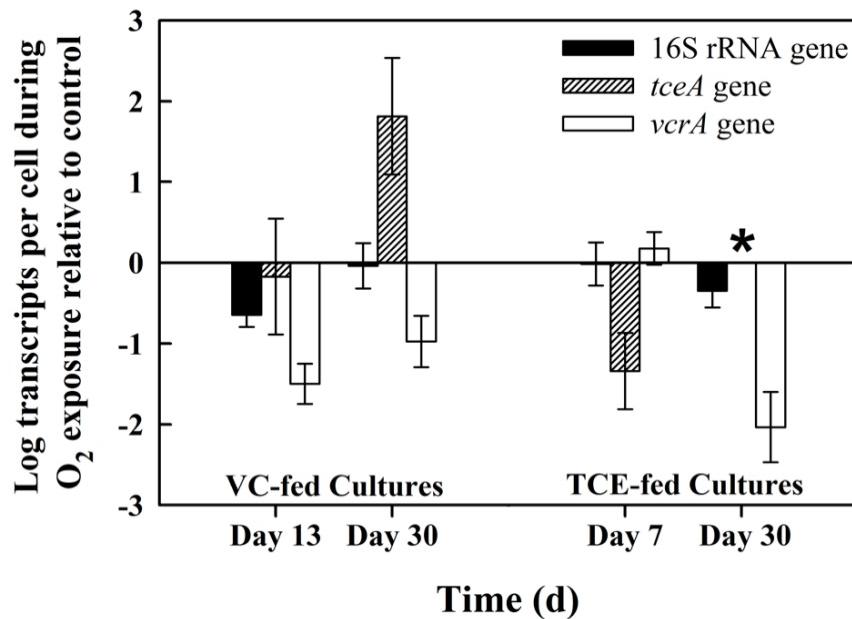
Figure 4.7. RT-qPCR analysis of the number of *Dhc* 16S rRNA, *tceA* and *vcrA* transcripts per cell in the oxygen-amended BDI cultures relative to the number of transcript copies per cell in the positive control cultures. Transcript copy numbers are normalized on a per cell basis (e.g., *tceA* gene transcripts divided by *tceA* gene copies). All data points represent average values from triplicate cultures, and error bars represent one standard deviation. The asterisk (*) indicates that *tceA* gene transcript copies were not detected in the oxygen-amended, TCE-fed cultures on Day 30. The lack of an error bar for this time point, therefore, does not indicate a 1:1 ratio of transcripts.

Table 4.1 presents the relative change in the number of transcripts per cell with time (i.e., the ratio of transcript copy numbers per cell to the number of transcript copies per cell in the inoculum). A ratio near unity (close to 0 in Table 4.1) indicates an insignificant change in the number of transcripts per cell. A ratio above unity (a positive number on the log scale) represents up-regulation of gene transcription, while a ratio below unity (a negative number on the log scale) indicates down-regulation of gene transcription. Normalizing gene expression to cell numbers may introduce a bias toward down-regulation of gene transcription since the total number of cells enumerated, both viable and nonviable, likely provide a higher estimate than the number of cells actively producing transcripts. As shown in Table 4.1, transcription of the *tceA* gene was down-regulated by 1.2 ± 0.2 to 2.0 ± 0.4 orders of magnitude in the VC-fed control cultures (Days 13 and 30, respectively). In contrast, the number of *vcrA* gene transcripts per cell

in the VC-fed control cultures increased by 0.7 ± 0.2 orders of magnitude (Table 10, Day 13) before returning to levels similar to those observed initially. In the TCE-fed control cultures, during TCE conversion to *cis*-DCE and VC, transcription of the *tceA* gene was initially up-regulated (2.1 ± 0.4 orders of magnitude; Table 4.1, Day 7). By the end of the incubation, the number of *tceA* gene transcripts per cell returned to levels similar to those observed initially (Table 4.1). Transcription of the *vcrA* gene in the TCE-fed control cultures was up-regulated at the onset of ethene production (0.5 ± 0.2 orders of magnitude; Figure 4.6 and Table 4.1, Day 7) and remained elevated in the presence of VC (2.0 ± 0.2 orders of magnitude; Table 4.1, Day 30).

Table 4.1. Summary of RT-qPCR analysis of *Dhc* 16S rRNA molecules and *tceA* and *vcrA* transcripts in the oxygen-amended and the positive control BDI cultures relative to the inoculum.

	Time (d)	Log Change in Transcripts [a]		
		16S rRNA	*tceA*	*vcrA*
VC-fed Cultures				
Oxygen-amended	13	-0.5 ± 0.2	-1.4 ± 0.7	-0.8 ± 0.2
	30	-0.05 ± 0.21	-0.2 ± 0.6	-0.9 ± 0.3
Positive control	13	0.2 ± 0.2	-1.2 ± 0.2	0.7 ± 0.2
	30	-0.01 ± 0.29	-2.0 ± 0.4	0.06 ± 0.18
TCE-fed Cultures				
Oxygen-amended	7	0.3 ± 0.2	0.8 ± 0.4	0.7 ± 0.2
	30	0.6 ± 0.1	ND [b]	-0.05 ± 0.40
Positive control	7	0.3 ± 0.3	2.1 ± 0.4	0.5 ± 0.2
	30	0.9 ± 0.2	0.2 ± 0.4	2.0 ± 0.2

[a] Log change in transcripts per cell (e.g., *tceA* gene transcripts divided by *tceA* gene copies) relative to the number of transcripts per cell in the inoculum

[b] ND, not detected

Dhc 16S rRNA and *tceA* and *vcrA* transcripts were also detected in the oxygen-amended VC- and TCE-fed cultures (Table 4.1). In contrast to the control (i.e., no oxygen) cultures, transcription of the *tceA* and *vcrA* genes did not correlate with dechlorination activity and seemed independent of the presence and/or absence of specific chlorinated

electron acceptors (Table 4.1). In the oxygen-amended, VC-fed cultures, transcription of the target genes was down-regulated (0.05 ± 0.21 to 1.4 ± 0.7 orders of magnitude; Table 4.1). The number of 16S rRNA molecules increased slightly over the period of 30 days although both *tceA* and *vcrA* transcripts were not detected or decreased after initially increasing in the oxygen-amended, TCE-fed cultures (Table 4.1). Figure 4.6 demonstrates that the ratios of gene transcripts per cell in the inactive cultures (i.e., oxygen-amended) relative to actively dechlorinating control cultures declined or were indistinguishable. In the VC-fed cultures, *tceA* gene transcripts per cell were actually detected at significantly higher levels per cell (1.8 ± 0.7 orders of magnitude) in the oxygen-amended as compared to the control cultures (Figure 4.6, Day 30). This finding does not indicate increased *tceA* transcription in the oxygen-amended cultures but rather a significant down-regulation of *tceA* transcription in the VC-fed control cultures (i.e., 2.0 ± 0.4 orders of magnitude at Day 30; Table 4.1).

Experiments with the PCE-dechlorinating consortia BDI and OW exposed to elevated temperatures corroborated that stressors affect RDase gene activity (37). Consortia BDI and OW produced ethene when incubated at temperatures of 30°C, but VC accumulated when cultures were incubated at 35 or 40°C. Incubation at 45°C resulted in complete loss of dechlorination activity. *Dhc* 16S rRNA, *bvcA*, and *vcrA* gene abundances in cultures showing complete dechlorination to ethene at 30°C exceeded those measured in cultures incubated at higher temperatures, consistent with the observed dechlorination activities and growth (Figure 4.8).

Conversely, biomarker gene transcript abundances per cell in cultures incubated at 35 and 40°C were generally at least one order-of-magnitude greater than those measured in ethene- producing cultures incubated at 30°C. Even in cultures accumulating VC, transcription of the *vcrA* gene, which is implicated in VC-to-ethene dechlorination, was up-regulated. These findings indicate that elevated temperatures result in increased transcription of *tceA* and *vcrA* (and possibly other RDase genes) but this increase did not correlate with reductive dechlorination activity (37).
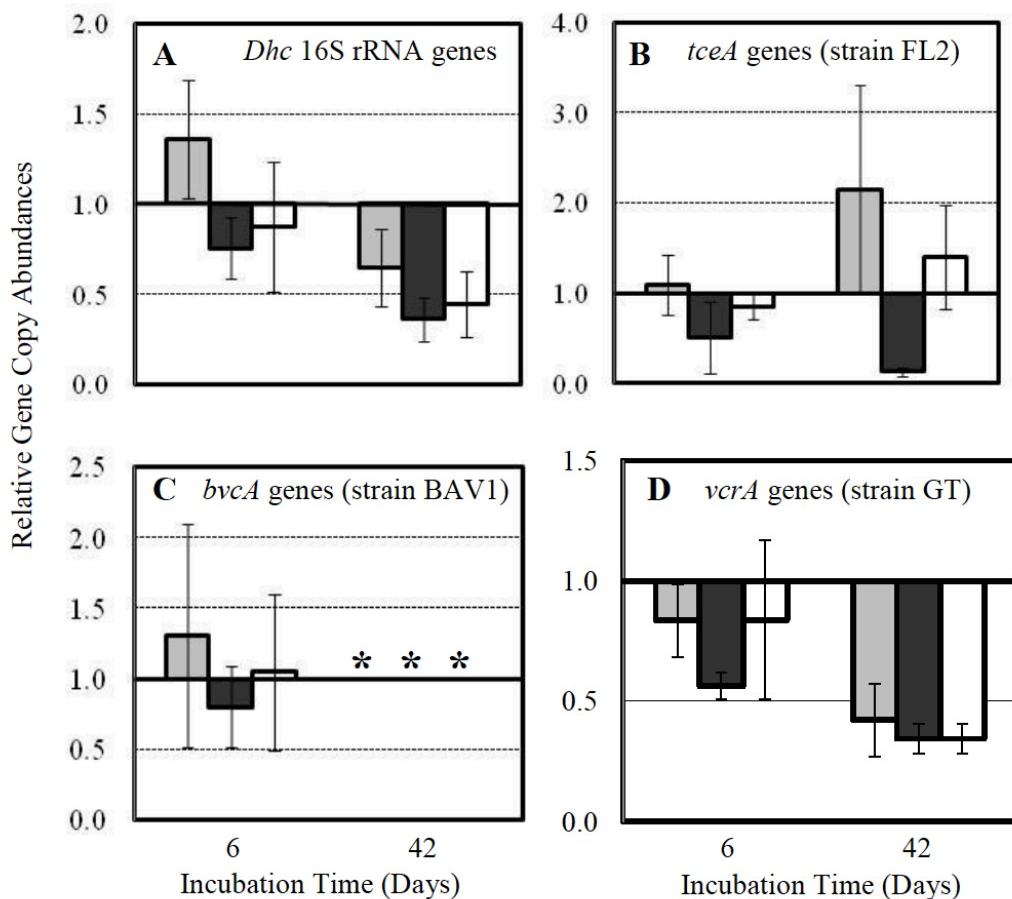
Figure 4.8. *Dhc* gene copy abundances in cultures incubated at 35ºC (gray bars), 40ºC (black bars), and in starved control cultures incubated at 30ºC (white bars). Abundances of *Dhc* 16S rRNA (A), *tceA* (B), *bvcA* (C), and *vcrA* (D) gene copies were normalized to quantities measured in cultures incubated at 30°C and amended with PCE. The asterisks indicate that the *bvcA* gene was below the detection limit of 9.5 x 10³ gene copies per mL culture fluid in cultures incubated at 35 and 40°C and in starved control

Regulation of RDase Gene Transcription in *Dhc*

The regulation of RDase gene expression in *Dhc* is not fully understood. While regulatory features have been identified on *Dhc* genomes (78) and up-regulation of RDase gene activity (i.e., transcription) in the presence of a suitable chlorinated electron acceptor has been demonstrated (110), the experiments discussed in this section revealed that RDase transcript turnover in *Dhc* can be slow (weeks to months) and that stressors affect RDase gene transcription. *Dhc* have been described as strict anaerobes and it was not surprising that oxygen exposure of BDI caused a loss of reductive dechlorination

activity. RDase gene transcripts remained quantifiable in oxygen-inhibited cultures that showed no reductive dechlorination activity. On a per cell basis, the number of *Dhc* 16S rRNA molecules and *tceA* and *vcrA* transcripts increased in oxygen- and TCE-amended, non-dechlorinating cultures (9). Similar observations were made in *Dhc*-containing consortia exposed to elevated temperatures (37). As discussed by Amos et al. (2008), "ghost" signals may be detected when targeting RDase gene transcripts. Transcripts produced under stress conditions do not correlate with the reductive dechlorination phenotype and represent a stress response.

Implications for RT-qPCR Data Interpretation at Bioremediation Sites

Understanding the effects of changing environmental conditions (e.g., oxygen, temperature, pH stress) on biomarker gene and transcript abundances and dechlorination activity is obviously important for meaningful interpretation of transcript abundance data generated with RT-qPCR. Apparently, stress conditions, such as oxygen exposure, elevated temperatures, starvation, and likely other stressors, influence *Dhc* RDase gene expression indicating that *Dhc* gene expression measurements must be interpreted cautiously as *Dhc* biomarker gene transcript abundances may not correlate with dechlorination activity. A more detailed understanding of the regulatory networks in organohalide-respiring *Chloroflexi* is desirable, so that gene-, transcript-, and protein-centric measurements can be meaningfully analyzed and interpreted, and brought into context with geochemical data and site remedial efforts.

Task 5: Fluorescence *In Situ* Hybridization (FISH) Approaches

This task evaluated the utility of ribosomal- and functional gene-targeted FISH approaches to visualize and quantify *Dhc* cells, elucidate their associations with other community members, and how these approaches can complement other MBTs, in particular qPCR. The benefit of FISH in comparison to other fluorescence microscopy based methods such as nucleic acid stains for total counts (e.g., DAPI) and activity targeted stains (e.g., Live Bac stain, Molecular Probes, Inc.) is that FISH enables specific identification of desired gene targets. As a non-PCR-based technique, FISH offers unique advantages and may complement qPCR tools to enumerate *Dhc* cell numbers. Although FISH has been successfully applied in a variety of environmental systems, FISH is rarely used for bioremediation monitoring.

The most common target for *in situ* identification of bacterial cells, including *Dhc*, by standard FISH is 16S rRNA (6, 11, 162). Unfortunately, standard FISH protocols and published 16S rRNA-targeted probes have drawbacks including (i) weak fluorescence signals and confounding background noise making the interpretation of FISH results challenging, in particular with environmental samples; (ii) the lack of specificity of the available probes (i.e., false positive results cannot be excluded); and (iii) the inability to distinguish *Dhc* strains with different dechlorination activities and RDase genes. CARD-FISH employing the same ribosomal *Dhc* FISH target sites as standard FISH can boost the fluorescence signal (5, 16, 27). Despite this advancement, only few studies have employed CARD-FISH successfully for routine screening of *Dhc* cells in environmental samples (26, 102). Compared to standard FISH protocols, CARD-FISH requires extensive expertise, and is time-consuming, expensive and less flexible because multiple probes have to be applied simultaneously for accurate identification of target cells.

A systematic approach was applied to explore if the *Dhc*-targeted FISH approach can be improved with respect to specificity and efficiency (e.g., time and labor). Specifically, we (i) aligned both the 16S rRNA genes and the 23S rRNA genes of *Dhc* and other dechlorinators to identify suitable probe target sites, (ii) evaluated refined FISH protocols

to visualize and quantify *Dhc* and other organohalide-respiring bacteria, (iii) compared FISH with qPCR, and (iv) explored other gene targets of interest, such as RDase genes.

Survey of Public Databases for 16S rRNA Gene Sequences of Dechlorinating
Chloroflexi

Additional target sites on the 16S rRNA and the 23S rRNA molecules were sought for designing a hierarchical set of FISH probes for accurate identification and quantification. For the database survey, 16S rRNA and 23S rRNA gene sequences of *Dhc* and other organohalide-respiring bacteria, as well as non-dechlorinating members of the *Chloroflexi*, were collected from public databases. In cases where isolates were available but sequence information was lacking (e.g., *Dhc* strain FL2), full-length 16S rRNA and 23S rRNA gene sequences were retrieved by standard cloning and sequencing techniques. Additional *Dehalococcoidia*-affiliated sequences were retrieved from 16S rRNA and 23S rRNA gene clone libraries established from genomic DNA extracted from PCE-dechlorinating mixed cultures and environmental samples. In addition, 16S/23S sequence databases were constructed for i) other known organohalide-respiring bacteria and some microbes commonly associated with dechlorinating enrichment cultures (i.e., *Sphaerochaeta*, methanogens and acetogens [e.g., *Acetobacterium* and *Syntrophobacter*]), and ii) environmental clone sequences retrieved from contaminated aquifers and subsurface environments.

***In Silico* Design of a 16S and 23S rRNA Multiple Probe Concept Within the Phylum**
Chloroflexi

Although the existing 16S rRNA-targeted FISH probe set (e.g., probes 1259c and 1259t (162)) is quite specific for *Dhc*, it lacks hierarchical organization. A more reliable identification of target the organism(s) can be achieved with the application of a hierarchical probe set consisting of multiple probes with phylum to the strain level specificity. Considering that the full diversity of organohalide respirers within the phylum *Chloroflexi* remains unknown, a hierarchic probe set may be especially useful for screening and identification of novel organohalide-respiring species and strains (especially if used in combination with functional gene probes as described below).

Figure 5.1 shows the application of a hierachical probe set to the phylum *Chloroflexi*. In addition to these new *Dhc*-targeted probes, hierarchical probe sets were constructed for other organohalide respirers, as well as *Sphaerochaeta*, methanogens and acetogens to possibly elucidate associations between these interacting community members.
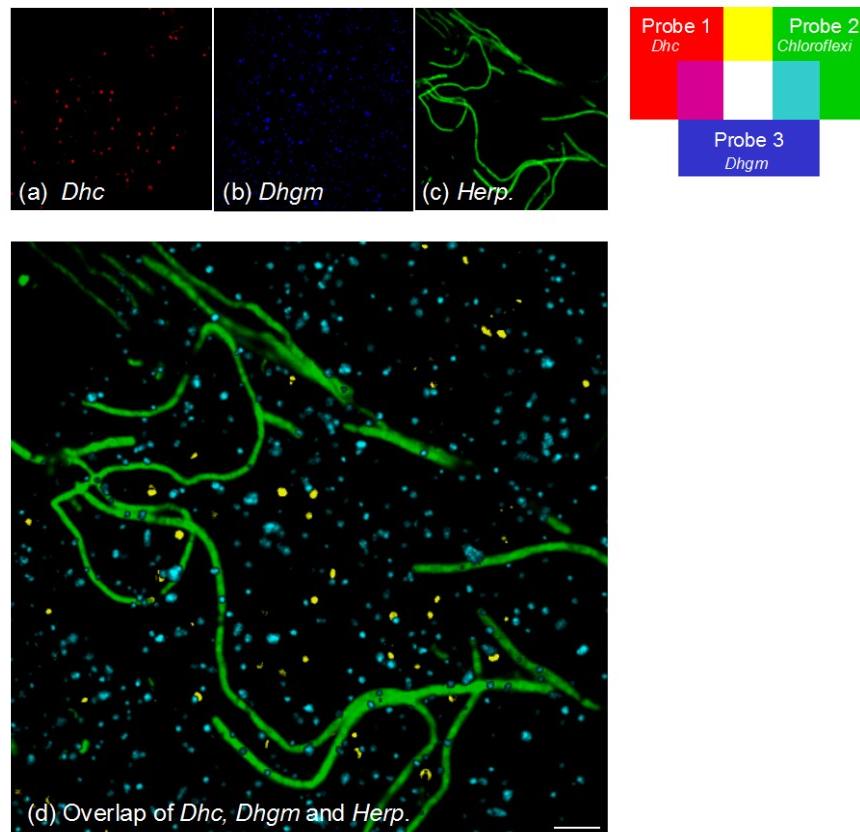
Figure 5.1. Probe combinations and colors demonstrating the principle of a ribosomal hierarchical probe set for three members of the phylum *Chloroflexi*. In this example, a defined mixture of pure cultures of members of the *Chloroflexi* including *Dhc* strain GT, *Dhgm* strain BLDC-9 and *Herpetosiphon aurantiacus* was used. *Dhc* (a) was targeted with the novel probe set labeled with Cy3 (red), *Dhgm* (b) was targeted with the novel probe set labeled with Cy5 (blue), and the non-dechlorinating, filamentous species *Herpetosiphon aurantiacus* (c) was targeted with the *Chloroflexi* phylum probe set labeled with FLUOS (green). In the composite image (d) with the phylum probe *Chloroflexi* (green), initially red *Dhc* cells appear yellow and initially blue *Dhgm* cells appear turquoise, whereas *Herpetosiphon* cells remain green. Bar equals 5 µm.

The ARB software package was used for identification and visualization of novel target sites for FISH probes (92, 93), targeting both 16S rRNA as well as the 23S rRNA sequences at different taxonomical levels (i.e., strain to phylum levels). Probe matches were performed against various public databases. The Delta G values and theoretical

formamide concentrations to predict theoretical optimal hybridization conditions were calculated using mathFISH (mathfish.cee.wisc.edu). These evaluations generated a set of probes for further experimental testing.

Application of rRNA-Targeted Probes to Visualize *Dhc* in Pure and Mixed Cultures
The standard rRNA-targeted oligonucleotide FISH techniques were tested with available reductively dechlorinating pure cultures and characterized, reductively dechlorinating consortia (BDI, OW, KB-1, TRS-1,2-DCP) under different growth conditions (e.g., different growth stages, stress conditions such as oxygen exposure). The purpose was to control and verify the specificity of various probes when used in combination. Optimization of the rRNA-targeted FISH approach also included testing of different biomass collection (i.e., centrifugation *versus* filtration), fixation and post sample treatment procedures to improve FISH results. These efforts demonstrated that stress factors like exposure of *Dhc* cells to oxygen (i.e., air) prior to fixation significantly reduced the FISH signals.

Using the standard ribosomal FISH approach, *Dhc* cells in actively dechlorinating cultures can yield satisfactory signals; however, the low ribosome content as well as their small cell size can make quantification challenging. CARD-FISH can enhance the fluorescent signal intensity but the approach suffers from other shortcomings that can lead to erroneous results interpretation (166).
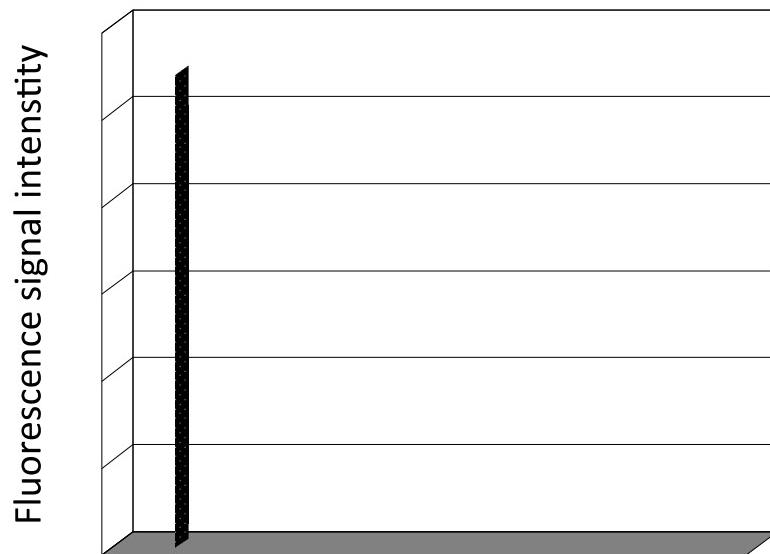
Figure 5.2. Effect of oxygen on FISH signal intensity. Upper panel: *Dhc* FISH signals decline significantly if the cells have been exposed to oxygen (air). Lower panel: Similar observations have been made with *Sphaerochaeta* cells, where the FISH signal intensity of oxygen-exposed *Sphaerochaeta* cells was significantly reduced.

In general, FISH results must be interpreted cautiously, in particular when environmental samples (e.g., groundwater) are analyzed. The application of a hierarchical probe set will introduce redundancy and control features, which will facilitate results interpretation but also increase the cost of the analysis. Further procedureal improvements to existing FISH protocols are possible to achieve assay flexibility (i.e., allowing the simultaneous use of several probes), to obtain strong probe signal intensities, and to decrease analysis cost and time.

FISH protocols have not been developed to target RDase genes, and attempts were made to explore if functional gene FISH can be developed for *Dhc* RDases. Several FISH protocols including mRNA CARD-FISH, polynucleotide FISH (also called RING-FISH) and *in situ* RCA (rolling circle amplification by PCR) FISH have been applied to target functional genes (151). The functional gene-targeted probes can be applied together with rRNA-targeted probes, concurrently providing phylogenetic and functional information. Figure 5.3 illustrates the simultaneous application of functional gene FISH and 16S rRNA-targeted FISH using defined cultures.
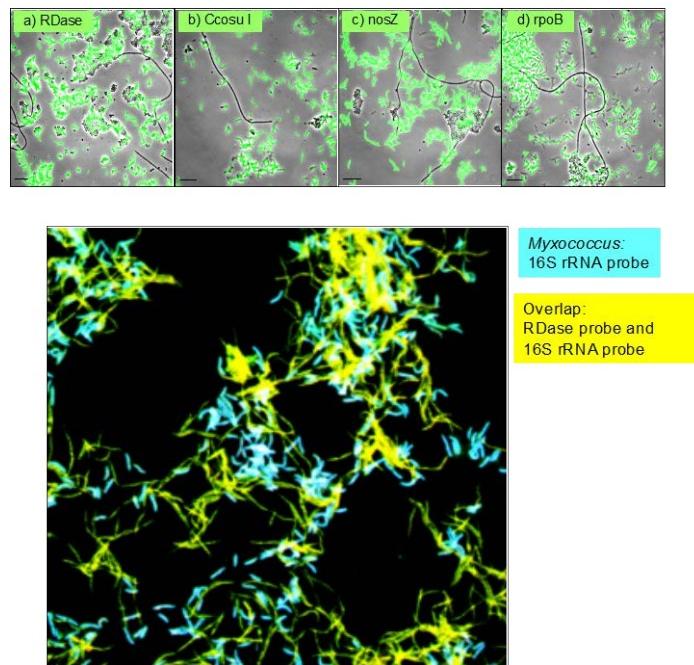
Figure 5.3. Demonstration of functional gene FISH and simultaneous application of 16S rRNA FISH using defined cultures. a-d) FISH targeting four different functional genes (*Adehal_0329, Adehal_0329*, *nosZ*, and *rpoB*) in a defined cell mixture: *Anaeromyxobacter dehalogenans, Herpetosiphon aurantiacus*, and *Staphylococcus aureus*. Bar equals 10 µm. e) Composite picture of 16S rRNA-targeted FISH and a functional gene FISH targeting the *Anaeromyxobacter dehalogenans*-specific RDase gene in a defined mixture of *Anaeromyxobacter dehalogenans* and *Myxococcus xanthus* cells.

Optimized FISH Protocols: Opportunities and Challenges

FISH can quantify target cells independent of biases introduced by nucleic acids extraction (i.e., DNA loss) and PCR (i.e., non-linear amplification). In addition, FISH is able to visualize and localize different cell populations to decipher possible interspecies interactions. For example, FISH could reveal juxtapositioning of *Dhc* with populations supplying nutrients (e.g., corrinoids, hydrogen). FISH allows specific enrichment of probe-targeted cells (e.g., via flow cytometry, polynucleotide FISH or magneto-FISH) for subsequent single cell-based procedures. On the flipside, published FISH protocols require extensive expertise to produce optimal results within cost and time constraints expected for routine analyses, especially when applied to environmental samples. FISH has therefore been applied to address fundamental microbiological questions with pure cultures. With more students and personnel trained in FISH, along with procedural advances, FISH could fill a niche to support environmental monitoring programs.

Task 6: Validate Biomarkers and Procedures Using Field Samples

Dhc biomarker gene-targeted qPCR is often applied at sites where natural attenuation or bioremediation are considered as treatment options for chlorinated ethene plumes and/or PCE/TCE source zones. A few field demonstration studies applying qPCR have been published (82, 83, 84, 91, 98, 122, 144); however, commercial laboratories such as Microbial Insights (www.microbe.com) perform the bulk of the site sample qPCR analyses, and these qPCR datasets have not been evaluated in a comparative fashion.

Site Data

Microbial Insights accumulated a large database with qPCR information for many sites that have undergone biostimulation, biostimulation combined with bioaugmentation, or no enhanced treatment. In addition, qPCR information of *Dhc* biomarker genes is available from a few sites where BioSep beads were used to collect microbial and chemical information, and from sites where chemical remedies were implemented. In a collaborative effort with Dora Ogles from Microbial Insights, we identified qPCR datasets from sites, for which chlorinated ethene and ethene concentration data and information about remedial treatment were available. Links to other site information were not established and all site names and site owners remained anomynous. From the selected pool of contaminant, ethene and qPCR data, correlation analysis was performed for 953 samples collected from 895 wells representing 62 sites undergoing monitored natural attenuation (MNA), bioremediation treatment, or chemical treatment. Concentrations of chlorinated ethenes and/or ethene were available for the majority of wells and sites sampled (726 wells, 54 sites). Excel was used to sort the data by treatment: biostimulation (200 wells, 23 sites), bioaugmentation (116 wells, 7 sites), MNA (584 wells, 54 sites), chemical oxidation (Chem Ox) (8 wells, 2 sites), pump and treat (8 wells, 2 sites) and zero valent iron (ZVI) (37 wells, 5 sites) (Table 6.1). Biomass was collected from groundwater on site using Sterivex cartridges or in the analytical laboratory via vacuum filtration as described (124). In addition, Bio-Sep® beads (www.microbe.com) were available from 72 wells. DNA was extracted as described (124) and *Dhc* biomarker genes and total bacterial 16S rRNA gene abundances were determined.

Table 6.1. Groundwater samples included in *Dhc* biomarker analysis. Also
indicated are the numbers of wells, for which qPCR data for *Dhc* and total bacterial 16S
rRNA genes were available. ND indicates not detected, while DNQ indicates that the
target genes were detected but were below the quantification limits.

| | | | | | | *Dhc* abundance | | |
Treatment	Samples (wells)	Sites	Bacteria detected	ND	DNQ	Low ($<10^3$ L^{-1})	Medium ($<10^7$ L^{-1})	High ($>10^7$ L^{-1})
Total wells	**953**	**62**	**418**	**103**	**119**	**209**	**380**	**142**
Bioaugmentation	116	7	116	10	6	21	28	51
Biostimulation	200	23	154	28	10	31	88	43
MNA	584	54	92	51	99	144	242	48
Chem Ox	8	2	8	0	0	2	6	*0*
ZVI	37	5	37	6	4	11	16	*0*
Pump and Treat	8	2	8	3	0	5	0	0

The *Dhc* abundance data were binned into the following categories: high at $>10^7$ L^{-1}
($>10^5$/bead); medium at 10^3-10^7 L^{-1} (10^2-10^5 per bead); low at $<10^3$. Gaps in terms of
compounds analyzed and gene targets sought remained in the data sets and prohibited a
rigorous principle component analysis; however, sufficient data for robust comparisons
of chlorinated ethenes, ethene, *Dhc* 16S rRNA genes and the RDase genes *tceA, bvcA,
vcrA* were available for the sites included in the analysis.

Groundwater Sampling, Biomass Collection, DNA Extraction, and qPCR
Groundwater sampling used low flow methods and collection occurred after geochemical
parameters had stabilized (124). Biomass was collected *on site* using Sterivex cartridges
or groundwater was shipped to the analytical laboratory and biomass was collected by
vacuum filtration onto 0.2 µm membrane filters. Sterivex cartridges were used for
biomass collection from groundwater at the majority (845) of wells (124). The volumes
of groundwater filtered were noted on each cartridge and shipped to the analytical
laboratory using SOPs (123, 124) (www.microbe.com). To ensure efficient DNA
extraction, different nucleic acid extraction procedures were initially compared for
robustness and for their ability to yield reproducible DNA yields, a prerequisite for
comparative data analysis. These evaluations determined that the MO BIO PowerSoil

Kit combined with a Powerlyzer 24 homogenizer (2,000 rpm, 5 min) cell lysis step yielded the best results in terms of DNA yield and ease of application (122, 123, 124). Appropriate quality control protocols were established from sample collection through qPCR analysis to ensure reproducibility (122). It is important to note that the sampling methodology used at a site or at specific wells was consistently applied, and groundwater/Sterivex cartridge handling, DNA extraction, and qPCR analysis followed the same procedures. Therefore, the qPCR analysis generated directly comparable datasets. To obtain biomass from Bio-Sep® beads, the beads were homogenized at 4,000 rpm for 45 sec using the Powerlyzer bead beater (microbial extraction protocol) and the Power Soil DNA extraction Kit (MO BIO) as described by the manufacturer. Protocols to quantify DNA and prepare qPCR calibration curves followed established procedures (122).

Dhc Biomarker Gene Abundances in Groundwater

Each groundwater sample was treated as an independent value, as current site characterization practice often limits molecular analysis to few wells along the contaminant flow path. These data are then used to determine possible treatment alternatives for a site. At many sites, temporal analyses of contaminant concentrations are collected to determine if cDCE and/or VC concentrations decline, which is often performed in lieu of ethene measurements. Alternatively, the increase in *Dhc* 16S rRNA, *bvcA* and *vcrA* gene copy numbers associated with the reductive dechlorination of polychlorinated ethenes and/or VC indicates that complete detoxification is possible.

Dhc were detected in 850 (90%) wells, and quantified in 731 (77%) wells. Furthermore, *Dhc* 16S rRNA genes were quantifiable at a majority of wells located in areas influenced by bioaugmentation (86%), biostimulation (81%) and MNA (74%) treatment (Table 6.1). Table 6.1 summarizes the abundance of *Dhc* biomarker genes in wells from sites or areas undergoing different treatments. Well locations influenced by bioaugmentation with commercially available consortia (116 wells) or by biostimulation only (200 wells) demonstrated higher numbers of *Dhc* 16S rRNA genes and the three RDase genes compared to sites without treatment. These observations corroborate that *Dhc* grow and

thrive using the chlorinated contaminants as substrates when appropriate environmental conditions are met.

Typically, individual *Dhc* RDase gene abundances were equal to or less than the total *Dhc* abundances. The *bvcA*, *vcrA* and *tceA* genes were quantified at $>10^3$ L^{-1} in 65%, 82% and 76%, at $>10^4$ L^{-1} in 34%, 62% and 63%, and at $>10^7$ L^{-1} in 4%, 32% and 33% of the bioaugmentation wells where *Dhc* were detected, respectively. At the majority of the bioaugmentation sites, the RDase genes dominating the inoculum also dominated the site post bioaugmentation. At biostimulation sites, the *bvcA*, *vcrA* and *tceA* genes were less abundant. Greater than 10^3 L^{-1} *bvcA*, *vcrA* and *tceA* genes were measured at 8%, 30% and 51% of the wells, respectively; 12%, 22% and 36% of the wells possessed $>10^4$ L^{-1}, and only 3%, 5% and 8% demonstrated abundances $>10^7$ L^{-1}. MNA sites had fewer wells with *bvcA*, *vcrA* and *tceA* gene abundances $>10^3$ L^{-1} (23%, 33%, and 16% of the wells, respectively), $>10^4$ *Dhc* L^{-1} (15%, 18% and 5%, respectively), and $>10^7$ L^{-1} (2%, 2%, and 3% of the wells, respectively).

Correlation of *Dhc* Biomarker Gene Abundances with Chlorinated Ethenes and Ethene Concentrations

The presence and concentrations of chlorinated ethenes were compared to *Dhc* biomarker gene abundances at sites undergoing different treatments. No significant bivariate correlations between *Dhc* 16S rRNA gene or RDase gene abundances were identified for PCE and TCE, suggesting involvement of other organisms, such as *Dhb* or *Geobacter*, in PCE and TCE reductive dechlorination. Although biomarkers for PCE-to-cDCE dechlorinators are not standard targets, our findings suggest that their inclusion in environmental monitoring programs allows for a better perspective of the site's reductive dechlorination performance capabilities (see Table 3.1).

Ethene presence correlated with *Dhc* abundance (Figure 6.1) and bivariate analysis indicated significant correlations between *Dhc* abundance and ethene concentration (n = 176, F = 74.8501, P = <0.0001, R2 = 0.3008). High *Dhc* abundances positively correlated with increasing amounts of ethene detected in 295 wells among the 704 samples obtained from wells with available ethene concentration data (Figure 6.1).

Of the 295 wells with ethene, 262 (89%), 129 (43%) and 155 (53%) tested positive for *Dhc* 16S rRNA, *bvcA* and *vcrA* genes, respectively, and were present at abundances $>10^5$ L^{-1} in approximately half of the wells (i.e., 103, 41 and 63 wells, respectively). Wells with *Dhc* biomarker genes at abundances $>10^5$ L^{-1} exhibited higher ethene concentrations (53 ppb ± 103 ppb, n=103) than those with $<10^5$ *Dhc* L^{-1} (6 ppb ± 17 ppb, n=127), thus supporting the bivariate correlations of *Dhc* 16S rRNA genes and the VC RDase genes *bvcA* and *vcrA* with ethene formation (Tables 6.2A and 6.2B).
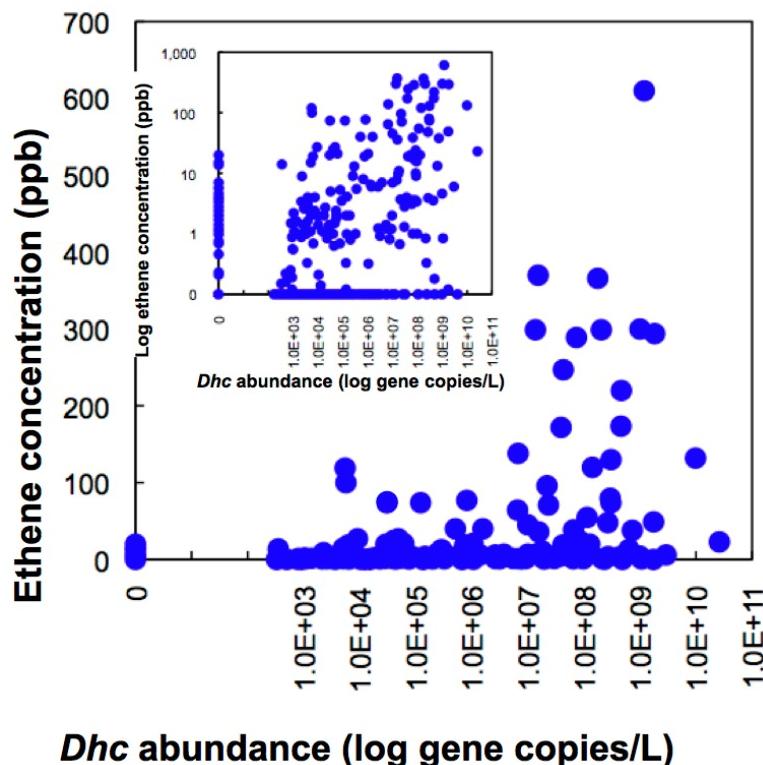
Figure 6.1. Correlation of ethene concentrations with *Dhc* abundances. The analysis included data from 625 wells where ethene was measured. "1.0 e+00" L^{-1} indicates the wells where *Dhc* was not detected. The inset shows the same data graphed on a log-log scale to highlight wells where some ethene was produced but no *Dhc* were detected. These wells were predominantly from sites undergoing ZVI treatment or MNA was implemented.

Positive correlations supported with statistical significance were also established between cDCE concentrations and *Dhc* 16S rRNA, *tceA* and *vcrA* gene abundances in groundwater samples. In addition, correlations were identified between VC concentrations and *Dhc* 16S rRNA, *tceA* and *vcrA* gene abundances.

In groundwater samples, *bvcA* abundances did not correlate with cDCE or VC concentrations; however, positive correlations between *bvcA* abundance and ethene formation were observed with the Bio-Sep® bead samples (see below). This may have been due to the composition of the bioaugmentation inoculum, as the beads had been inoculated with consortium BDI. BDI provides a combination of the three *Dhc* strains BAV1, GT and FL2, and strain BAV1 harbors the *bvcA* gene. The *vcrA* gene abundances did not correlate with ethene formation in the bead samples suggesting that *bvcA* was a more reliable indicator of detoxification at these particular sites where Bio-Sep® beads were used.

In summary, the analysis of the qPCR and ethene datasets supported that ethene formation in groundwater generally correlates with the presence of *Dhc* (50, 57), and also is commonly observed when *Dhc* abundances exceed 10^7 L^{-1} (90, 91). Ethene formation was also associated with an increased abundance the *vcrA* and *bvcA* RDase genes implicated in cDCE- or VC-to-ethene dechlorination at about half of the wells corroborating the findings of a prior study (90).

Table 6.2. Bivariate analysis comparing the concentrations of individual chlorinated ethenes to *Dhc* abundance in (A) groundwater and (B) associated with Bio-Sep® beads. Significant correlations for groundwater samples with a P value < 0.005 are shown in blue bold font. Weak correlations have a P value of <0.08. For Bio-Sep® beads, P values of < 0.02 are shown in bold.

(A) Groundwater

Pairwise Comparison	Observations	F value	P value	R^2	Significance
PCE to *Dhc*	312	0.1399	0.709	0.00045	no
PCE to *tceA*	142	0.0558	0.814	0.000396	no
PCE to *bvcA*	82	2.8515	0.095	0.034417	no
PCE to *vcrA*	126	0.0005	0.982	0.000004	no
TCE to *Dhc*	380	0.0062	0.937	0.000016	no
TCE to *tceA*	198	0.5416	0.463	0.002756	no
TCE to *bvcA*	114	0.0384	0.845	0.000343	no
TCE to *vcrA*	140	0.0718	0.789	0.00052	no
1,1-DCA to *Dhc*	**154**	**0.1119**	**0.074**	**0.000736**	**weak**
1,1-DCA to *tceA*	78	1.1724	0.282	0.015192	no
1,1-DCA to *bvcA*	26	1.2693	0.271	0.050232	no
1,1-DCA to *vcrA*	26	0.5026	0.485	0.020512	no
tDCE to *Dhc*	72	0.1345	0.715	0.001918	no
tDCE to *tceA*	50	0.0039	0.951	0.000081	no
tDCE to *bvcA*	**29**	**3.5535**	**0.070**	**0.116306**	**weak**
tDCE to *vcrA*	28	0.0535	0.819	0.002055	no
cDCE to *Dhc*	**338**	**37.6523**	**<.0001**	**0.100768**	**yes**
cDCE to *tceA*	**198**	**10.9785**	**<0.0011**	**0.053042**	**yes**
cDCE to *bvcA*	109	0.0989	0.754	0.000924	no
cDCE to *vcrA*	**136**	**3.394**	**0.068**	**0.024703**	**weak**
VC to *Dhc*	**238**	**75.3012**	**<0.0001**	**0.241892**	**yes**
VC to *tceA*	**173**	**64.5744**	**<0.0001**	**0.274115**	**yes**
VC to *bvcA*	84	0.1949	0.660	0.002372	no
VC to *vcrA*	**112**	**11.6951**	**<0.0009**	**0.96101**	**yes**
ethene to *Dhc*	**176**	**74.8501**	**<0.0001**	**0.300784**	**yes**
ethene to *tceA*	**108**	**73.2656**	**<0.0001**	**0.408698**	**yes**
ethene to *bvcA*	**77**	**9.8926**	**0.002**	**0.116531**	**yes**
ethene to *vcrA*	**110**	**71.9905**	**<0.0001**	**0.399968**	**yes**

(B) Bio-Sep® Beads

Pairwise Comparison	Observations	F value	P value	R^2	Significance
PCE to *Dhc*	18	3.6831	0.073	0.187118	no
PCE to *tceA*	8	5.6372	0.055	0.48441	no
PCE to *bvcA*	10	5.2683	0.051	0.397057	no
PCE to *vcrA*	5	5.2119	0.107	0.634677	no
TCE to *Dhc*	19	0.872	0.364	0.048793	no
TCE to *tceA*	10	1.3381	0.281	0.143291	no
TCE to *bvcA*	11	0.158	0.700	0.017255	no
TCE to *vcrA*	8	0.0398	0.848	0.006592	no
cDCE to *Dhc*	57	1.8431	0.180	0.032424	no
cDCE to *tceA*	38	0.2786	0.601	0.007667	no
cDCE to *bvcA*	38	1.0883	0.304	0.029343	no
cDCE tp *vcrA*	34	1.058	0.311	0.032005	no
VC to *Dhc*	**35**	**17.2963**	**<0.00001**	**0.343888**	**yes**
VC to *tceA*	**28**	**13.6407**	**0.001**	**0.344109**	**yes**
VC to *bvcA*	**27**	**6.9106**	**0.014**	**0.216561**	**yes**
VC to *vcrA*	**24**	**6.7682**	**0.016**	**0.235268**	**yes**
ethene to *Dhc*	**61**	**22.9258**	**<0.00001**	**0.279836**	**yes**
ethene to *tceA*	**41**	**5.8581**	**0.020**	**0.130592**	**yes**
ethene to *bvcA*	**42**	**9.7342**	**0.003**	**0.195725**	**yes**
ethene to *vcrA*	37	2.0281	0.163	0.054772	no

Measurable Indicators of Ethene Formation

Data were obtained for both bacterial and *Dhc* 16S rRNA gene abundances from 418 wells, 184 of which produced ethene. The highest ethene concentrations were observed in wells where the *Dhc* 16S rRNA genes represented >0.05% of the total bacterial 16S rRNA gene abundance (Figure 6.1). In other words, the *Dhc*-to-total-bacterial 16S rRNA gene ratio correlated with ethene formation in these 184 wells, suggesting that ratios of >0.0005 provide a measurable indicator for detoxifcation (Figure 6.2).
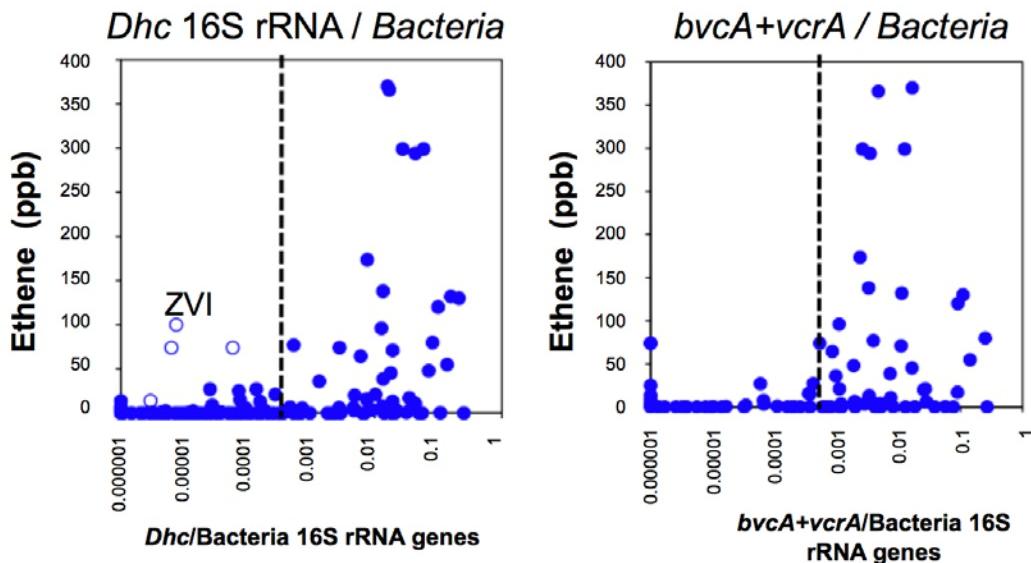
Figure 6.2. Ethene concentrations graphed against the *Dhc* 16S rRNA gene abundance as a portion of the bacterial 16S rRNA gene abundance (left) and against the *bvcA+vcrA*-to- bacterial 16S rRNA gene ratio (right). A "1" designates an equal abundance of *Dhc* to bacterial 16S rRNA genes. The open symbols in panels A and B point to samples that were identified as being in ZVI treatment zones. A *Dhc* abundance >0.0005-0.001 (0.05-0.1%) to the bacterial 16S rRNA gene abundance (dashed vertical lines) correlates with ethene and VC formation.

The ratio of the sum of the VC RDases *vcrA* and *bvcA* to total bacteria (Figure 6.2-right) mirrored the ratio of *Dhc* to bacteria (Figure 6.2-left) in wells where ethene was produced. These observations supported that VcrA and BvcA were the key VC RDases contributing to ethene formation at these sites. At two wells (open diamonds in Figure 6.2-left) ethene was formed and *Dhc* are present, but *bvcA* and *vcrA* were not detected suggesting that additional VC RDase genes may exist. The absence of *tceA* in these samples further supports the existence of yet unidentified VC RDase genes.

Furthermore, when the ratio of the sum of the VC RDases *vcrA+bvcA*-to-*Dhc* 16S rRNA genes was plotted against the ethene concentrations, the highest measured ethene concentrations were observed when the ratios of *bvcA+vcrA*-to-*Dhc* 16S rRNA genes were between 0.05 and 10. This observation at 224 wells suggested that the predominant

Dhc strains possess *vcrA* or *bvcA* at bioaugmentation wells. VC concentrations also correlated with the *bvcA+vcrA* gene-to-*Dhc* 16S rRNA gene ratios ranging between 0.05 to10 (Figure 6.3-left). Several wells with high VC concentrations also showed high abundances of *tceA*, but no detection of either *vcrA* or *bvcA* genes (boxed in Figure 6.3 right), TceA was characterized as TCE-to-VC RDase indicating that the abundance of *tceA* in the absence of VC RDase genes is a scenario that likely results in VC accumulation.
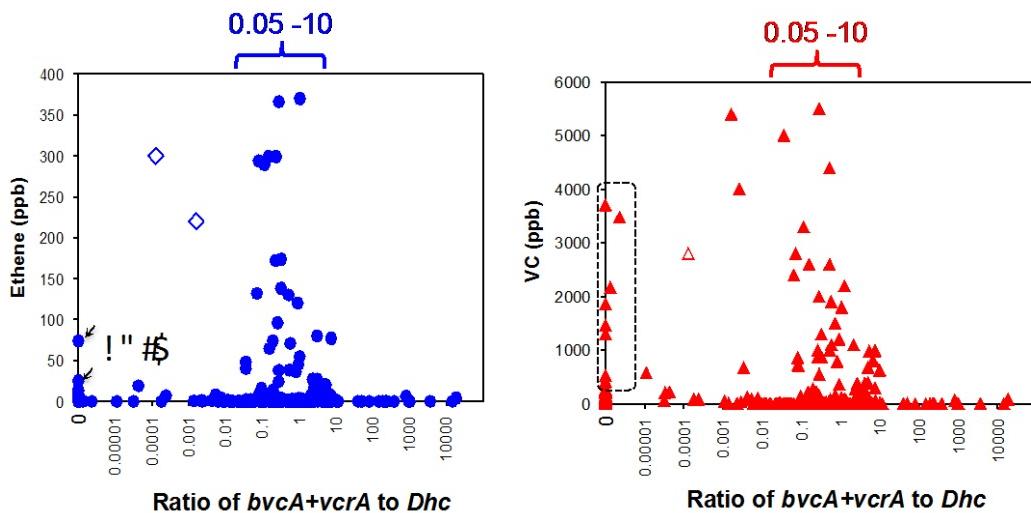
Figure 6.3. Ethene (right) and VC (left) graphed against the sum of the VC-RDases *bvcA* and *vcrA* gene displayed as a proportion of *Dhc* 16S rRNA gene abundance (x-axis). Small arrows on the right panel, indicate ZVI treatments, OPEN diamonds represent two MNA sites with low abundances of VC RDases, but high production of ethene. In the right panel, square dashed box surrounds wells without VC RDases, but with high *Dhc* 16S rRNA and *tceA* genes.

RDase Abundances Exceed *Dhc* Cell Numbers

At several wells, individual RDase genes outnumbered the total *Dhc* 16S rRNA gene copies by 5 up to 10,000-fold (Figure 6.4). Wells with more VC RDase gene copies than *Dhc* 16S rRNA genes did not exhibit higher ethene formation (Figure 6.3-left). Even with RDase gene abundances exceeding the *Dhc* abundance by 10,000-fold, no additional ethene formation was observed, suggesting that the extra RDase gene copies were not contributing to reductive dechlorination activity. In axenic *Dhc* cultures, a particular

RDase occurs in a 1:1 ratio with the *Dhc* 16S rRNA gene, as indicated in Figure 6.4 by the solid black line. Any point above this line represents samples with more copies of a particular RDase gene relative to the *Dhc* 16S rRNA gene copies.
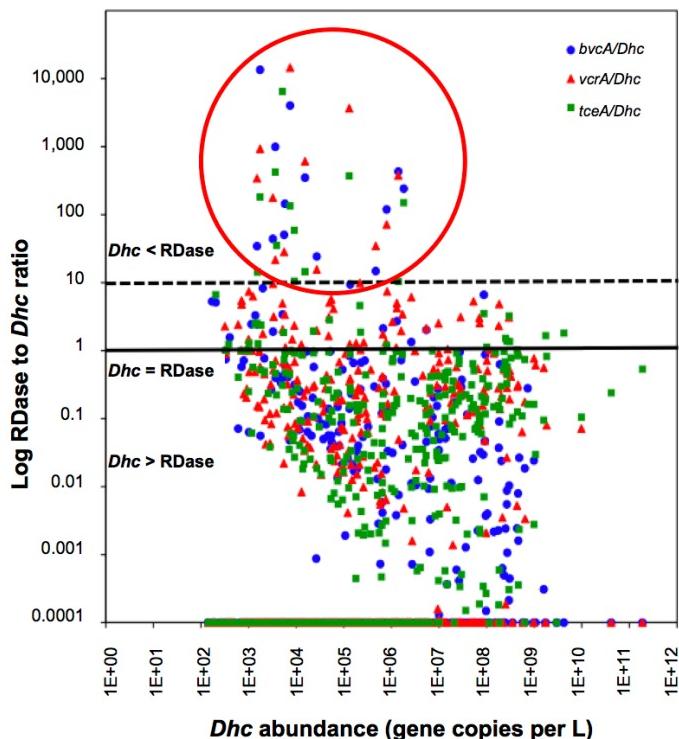
Figure 6.4. Samples from wells with more *bvcA*, *vcrA* and *tceA* genes than predicted by *Dhc* 16S rRNA gene abundance. The solid black line indicates a 1:1 ratio of RDase to *Dhc* 16S rRNA genes, the points above the dashed line (in the red circle) represent wells with a 10-fold or greater higher abundance of RDase genes.

A 2-3 fold difference could possibly be explained by qPCR standard curve viability or an RDase gene duplication. The dashed line in Figure 6.4 represents 10-fold higher abundance of a specific RDase gene relative to *Dhc*. A 10 to 10,000-fold excess of the *tceA* gene was observed in 14 samples collected from bioaugmentation (7) and MNA (7) treatment wells, while excess *bvcA* and *vcrA* genes were found in samples from 13 and 12 MNA wells, respectively. These observations suggest an unidentified reservoir for these RDase genes, presumably in a non-*Dhc* host. We considered that *Dhc* RDase genes could be harbored on the genomes of other organohalide-respiring *Chloroflexi*, such as *Dhgm*. Screening of samples with *Dhgm* 16S rRNA gene-specific qPCR assays indicated

that these bacteria did not comprise a significant portion of the microbial community at any of these sites, and did not represent a potential source of these excess RDase genes.

Dehalococcoides Attached to Bio-Sep® Beads

Although the set of data for the Bio-Sep® bead samples with attached biomass was limited to 78 wells, the data were congruent with those obtained with suspended biomass collected from groundwater (875 wells). The statistical analysis demonstrated limited differences between the datasets obtained with attached vs. suspended biomass samples. Bioaugmentation wells typically outperformed biostimulation-only wells in terms of ethene production and both Sterivex and and Bio-Sep® bead samples linked ethene formation with *Dhc* biomarker gene abundances. Because BioTraps (Bio-Sep® beads) were not deployed at any of the MNA sites, qPCR data from MNA sites obtained with suspended biomass could not be compared directly. Of note, the attached community did not exhibit the overabundance of RDase genes in excess of *Dhc*, and in only one well did the RDase genes exceed the *Dhc* cell number by 3- to 9-fold. Finally, the bead samples demonstrated a significant correlation of VC and ethene to *bvcA*. In the suspended biomass samples, a correlation was found between VC and ethene and *vcrA*. A possible explanation is that the bead samples were inoculated with consortium BDI, which provides a combination of three *Dhc* strains (BAV1, GT and FL2), and strain BAV1 harbors the *bvcA* gene. *Dhc* strain BAV1 grows with all DCE isomers and, depending on the environmental conditions, may dominate the *Dhc* community. The *vcrA* gene abundances did not correlate with ethene formation in the bead samples suggesting that *bvcA* was a more reliable indicator of detoxification at these particular sites where Bio-Sep® beads were used.

Biomarker Gene Abundance Ratios as a Predictor of Ethene Formation

Wells demonstrating effective dechlorination and ethene formation typically had $>10^5$ *Dhc* L^{-1} and had *Dhc* at $>0.05\%$ of total bacterial 16S rRNA gene abundance. In the 57% of wells, in which *Dhc* contributed $>0.01\%$ of the bacterial 16S rRNA genes, 86% of the wells produced ethene. Highly correlated with reductive dechlorination and ethene formation was a *Dhc* abundance $>10^6$ L^{-1}, and one or both VC RDase genes present

within an order of magnitude of the *Dhc* cell numbers. A lack of complete dechlorination (stalling at cDCE or VC) is correlated with a dearth of both *Dhc* 16S rRNA genes (<0.01% of the bacterial community) and VC RDase genes that occur at less than 0.1% of the *Dhc* abundance. Therefore, enumerating *Dhc* cells using 16S rRNA gene-targeted and RDase gene-targeted qPCR can provide information about a site's potential for ethene formation. Ideally, this measurement is normalized to another quantifiable parameters to generate a robust measurement that can be compared between wells and sites.

The analysis indicated that the following ratios of biomarker gene abundances strongly correlate with ethene formation at sites impacted with chlorinated ethenes.

1. *Dhc*-to-total bacteria 16S rRNA gene ratios greater than 0.0005 (0.05%) correlate with ethene formation (i.e., detoxification).
2. *bvcA+vcrA*-to-*Dhc* 16S rRNA genes ratios between 0.05 and 10 correlate with ethene formation (i.e., detoxification).

Because contemporary qPCR tools can measure these genes, these ratios are easy to establish and are useful to predict ethene formation.

Competition between *Dhc* Strains with *vcrA* vs *bvcA*

The *vcrA* gene was more abundant than the *bvcA* gene at the bioaugmentation sites. Bioaugmentation consortia used for inoculation are generally maintained with TCE as the electron acceptor. Under these conditions, *Dhc* strains with *vcrA* will outnumber *Dhc* strain BAV1-type populations carrying *bvcA*, which cannot grow with TCE. Among sites that were inoculated with consortium KB-1, more wells were enriched in *vcrA* than *bvcA*, in particular when the primary contaminant at the site was TCE. At one TCE-contaminated site that was inoculated with BDI, *bvcA* remained at a higher portion relative to *vcrA*. At MNA and biostimulation sites, *bvcA* and *vcrA* appear equally often, further suggesting that the inoculum determines the predominance of *vcrA* at bioaugmentation sites. Interestingly, there was a statistically significant correlation between the tDCE abundance and the *bvcA* gene (Table 6.2). *Dhc* strain BAV1 carrying *bvcA* can respire all DCE isomers, including tDCE, and possible outcompete *Dhc* strains

carrying *vcrA* under certain environmental conditions (14, 39, 82). Strain GT, which carries the *vcrA* gene, did not use tDCE as an energy source (86).

Summary of Field Data Analysis

Ethene formation was highest in wells with a *vcrA+bvcA*-to-*Dhc* 16S rRNA gene ratio near unity. In cases where RDase gene copies exceeded the *Dhc* cell abundance, no additional ethene formation was observed. Other useful measures that correlated with ethene formation is the ratio of *Dhc* 16S rRNA genes-to-total bacteria 16S rRNA genes and the ratio of *vcrA+bvcA*-to-total bacteria 16S rRNA genes. When ratios greater 0.0005 (0.05%) are determined in groundwater, ethene formation is likely. Ethene formation and the abundances of all *Dhc* biomarker genes correlated most strongly with bioaugmentation treatment. In a few wells, ethene formation occurred and *Dhc* were present, but none of the known *Dhc* RDase genes were detected, indicating the existence of other, not yet identified VC RDase genes. Weak or no correlations were seen between PCE and TCE reductive dechlorination and *Dhc* biomarker genes, suggesting these activities are likely contributed by other organohalide-respiring bacteria. Wells with low or no *Dhc* 16S rRNA or RDase genes also occurred where ethene was detected, in particular when undergoing ZVI treatment.

Overall, these findings corroborate the value of *Dhc* biomarkers as prognostic and diagnostic monitoring tools, and suggest that the ratio of *Dhc*/*Bacteria* is a simple and useful normalized measurement to predict detoxification (i.e., ethene formation); however, the analysis also demonstrated that comprehensive process understanding has not been obtained. In addition to monitoring for *Dhc* 16S rRNA genes and and RDase genes, other organisms and genes also should be considered, especially at mixed contaminant sites (see Chapter 2). Combining the information from population analysis with site geochemical properties is important to draw meaningful conclusions as to the fate of priority pollutants, in particular as they pertain to a stall in contaminant detoxification.

Task 7: Develop a Guidance Document that Clarifies Tools Application and Results Interpretation

Guidance documents for the application of MBTs for monitoring of relevant nucleic acid biomarkers in environmental samples have been prepared by the Interstate Technology Research Council (ITRC) Environmental Molecular Diagnostics (EMD) team. Dr. Löffler is an EMD team member and contributed to the preparation of Facts Sheets and other publically available documents (www.itrcweb.org/Team/Public?teamID=3). The results and lessons learned during the completion of SERDP ER-1586 are reflected in the ITRC documents, and a separate guidance document has not been prepared. Most of the data generated in ER-1586 are available in the public domain (e.g., peer-reviewed journal articles, as well as abstracts, posters and slides presented at workshops and symposia).

The following list summarizes the key findings of SERDP ER-1561 that are of value for remediation practice.

- The value of quantitative real-time PCR (qPCR) for monitoring biomarker genes (and transcripts) in environmental samples (i.e., groundwater) was documented. qPCR detects and enumerates organisms and genes across eight orders of magnutide in abundance, and is the method of choice for most environmental applications. Multiplex qPCR approaches are not recommended for environmental samples unless a priori knowledge of target gene abundances is available. Other MBTs such as microarray or FISH analyses can play a supporting role by supplying additional comparative information.

- TaqMan-based qPCR provides higher sensitivity (avoids false negatives) and higher specificity (avoids false positives) than SYBR Green-based assays for biomarker analysis in environmental samples.

- Standard operating procedures (SOPs) for biomass recovery from groundwater, nucleic acid extractions and qPCR analysis have been established (intregrated effort with ER-1561).

- New biomarker genes encoding specific RDases were identified and qPCR assays were designed and validated.

  cfrA: CF → DCM and 1,1,1-TCA → 1,1-DCA

  dcrA: 1,1-DCA → CA

  dcpA: 1,2-DCP → propene

- The underpinning mechanisms of reciprocal inhibition of cDCE and VC reductive dechlorination by CF and 1,1,1-TCA have been elucidated. Inhibition constants (K_i values) have been determined and will help identify sites where inhibition is likely to occur.

- The laboratory evaluation of culture blends suggests that inhibition of cDCE and/or VC reductive dechlorination by other chlorinated solvents such as CF and 1,1,1-TCA can be relieved.

- *Dhc*-to-total bacteria 16S rRNA gene ratios greater than 0.0005 (0.05%) correlate with ethene formation (i.e., detoxification).

- The available qPCR tools generate information about *Dhc* and total bacteria 16S rRNA gene abundances evoking these ratios as useful measures to predict ethene formation at sites impacted with chlorinated ethenes.

It is important to note that the MBT development has not reached its final stage, and significant further advancements are within reach. The pace, at which gene sequence information is being linked to specific functions, will continue to increase, and larger sets of biomarker genes for monitoring the detoxification of different groups of DOD-relevant contaminants will become available. Further, the technology used to collect biomarker information to decipher the microbiology controlling contaminant degradation and detoxification processes will advance rapidly. Consequently, larger datasets will be available for site assessment, monitoring programs and decision-making processes. For example, high-throughput sequencing of a composite of genes and/or transcripts associated with a groundwater sample can now be accomplished within days at

reasonable costs. Although the sequence data analysis is cumbersome and laborious with the current computational approaches, these bioinformatics tools will continue to improve rapidly, thereby reducing analysis time and costs. From a practical point of view, a key question is "How much information is deemed sufficient or necessary, and at what point does additional information no longer add value to the decision-making process?". The overaching premise is that MBT information will assist the selection of sites suitable for MNA or bioremediation treatment, and efficient bioremediation technology implementation is achieved in a sustainable manner (e.g., without causing secondary negative impacts on groundwater quality). From a scientific viewpoint, the value of MBTs to accomplish these goals is indisputable; however, parts of the practicing community are reluctant to use MBT data to support decision-making. Remedial efforts at many sites still rely on empirical practices, and the potential costsavings through consequent MBT application are not realized. MBT analysis is not free but reflects a rather insignificant contribution to the overall budget of a remediation project. There are several possible reasons why MBT data are not appropriately valued by sectors of the practicing community, one of which is the lack of understanding regarding the information MBTs provide, and the ensuing reluctance of changing established procedures. Another issue is that the interpretation of MBT data is not always straightforward and depending on the person(s) performing the analysis, the conclusions can differ. For example, insufficient MBT data collection at inappropriate well locations can complicate results interpretation. Further, MBT data should be considered in context with site geochemical and hydrogeologic information and site history (e.g., prior remediation efforts). Obviously, appropriate data interpretation is a critical issue and should receive adequate attention to ensure that proper conclusions ensue following the MBT analysis. As the accuracy and informational content of MBTs continue to increase, and integrated site data analyses procedures become standard protocol, the arguments for not rigorously applying MBTs to deliver the most efficient and economical site remedies should decline. Careful documentation of MBT-based decision-making, remedial activities, and site closures and will ultimately reveal the signicant cost-savings that can be realized through consequent MBT application.

Drs. Ritalahti, Edwards and Löffler remain committed to efforts aiding the transitioning of research findings to the practicing community. Practitioners regularly contact Drs. Ritalahti, Edwards and Löffler to answer specific questions about MBT application and results interpretation, a service we will continue to provide. The ER-1586 team will continue to attend meetings (Battelle conferences) focused on contaminant remediation and offer shortcourses focused on MBT application to support remedial efforts, as appropriate, to practitioners and regulators.

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Deliverables

Peer-Reviewed Manuscripts

1. **Padilla-Crespo, E., J. Yan, C. Swift, D. D. Wagner, K. Chourey, K. M. Ritalahti, R. L. Hettich and F. E. Löffler**. 2013. Identification and environmental distribution of the 1,2-dichloropropane-to-propene reductive dehalogenase *dcpA*. *Applied and Environmental Microbiology*. In Press.

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19. **McMurdie, P.J., L.A. Hug, E.A. Edwards, S. Holmes, and A.M.Spormann.** 2011. Site-specific mobilization of vinyl chloride respiration islands by a mechanism common in *Dehalococcoides*. BMC Genomics, 12:287

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24. **Grostern, A., M. Duhamel, and E.A. Edwards.** 2009. Chloroform respiration to dichloromethane by a *Dehalobacter* population. *Environmental Microbiology*. 12:1053-1060.

25. **Grostern, A., W.W.M. Chan, and E.A. Edwards.** 2009. 1,1,1-trichloroethane and 1,1-dichloroethane reductive dechlorination kinetics and co-contaminant effects in a *Dehalobacter*-containing mixed culture. *Environmental Science and Technology*. 43:6799-6807.

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32. **Fletcher, K.E., K.M. Ritalahti, B.K. Amos, K.D. Pennell, and F.E. Löffler.** 2008. Resolution of culture *Clostridium bifermentans* DPH-1 into two populations: A *Clostridium* sp. and tetrachlorethene (PCE) dechlorinating *Desulfitobacterium hafniense* strain JH1. *Applied and Environmental Microbiology*. 74:6141-6143.

33. **Strycharz, S.M., T.L. Woodard, J.P. Johnson, K.P. Nevin, R.A. Sanford, F.E. Löffler, and D.R. Lovley.** 2008. Graphite electrode as a sole electron donor for reductive dechlorination of tetrachlorethene by *Geobacter lovleyi*. *Applied and Environmental Microbiology*. 74:5943-5947.

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36. **Amos, B.K., J.A. Christ, L.M. Abriola, K.D. Pennell, and F.E Löffler.** 2007. Experimental evaluation and mathematical modeling of microbially-enhanced tetrachloroethene (PCE) dissolution. *Environmental Science and Technology*. 41:963-970.

37. **Krajmalnik-Brown, Y. Sung, R., K.M. Ritalahti, F. Michael Saunders, and F.E. Löffler.** 2007. Environmental distribution of the trichloroethene reductive dehalogenase gene (tceA) suggests lateral gene transfer among *Dehalococcoides*. FEMS Microbiology Ecology 59:206-214.

Peer-Reviewed Book Chapters

1. **Löffler, F.E., K.M. Ritalahti, and S.H. Zinder.** 2013. *Dehalococcoides* and reductive dechlorination of chlorinated solvents. In H.F. Stroo, A. Leeson and C.H. Ward (Eds.) *Bioaugmentation for Groundwater Remediation, SERDP ESTCP Environmental Remediation Technology,* Volume 5, pp. 39-88. Springer, New York.

2. **Hug, L.A., E.A. Edwards, H. Vrionis, and D.W. Major.** 2013. Research Needs: Bioaugmentation for Groundwater Remediation. In H.F. Stroo, A. Leeson and C.H. Ward (Eds.) *Bioaugmentation for Groundwater Remediation, SERDP ESTCP Environmental Remediation Technology,* Volume 5, pp. 333-362. Springer, New York.

3. **Lee, N.M., D.B. Meisinger, M. Schmid, M. Rothballer, and F.E. Löffler.** 2011. Fluorescence *in situ* hybridization in Geomicrobiology. In H.J. Reitner and V. Thiel (Eds.) *Encyclopedia of Geobiology*, pp. 854-880. Springer-Verlag, New York.

4. **Ritalahti, K.M., C. Cruz-García, E. Padilla-Crespo, J.K. Hatt, and F.E. Löffler**. 2010. RNA extraction and cDNA analysis for quantitative assessment of biomarker transcripts in groundwater, pp. 3671-3685. In K.N. Timmis (Ed.), *Handbook of Hydrocarbon and Lipid Microbiology.* Springer, Berlin.

5. **Ritalahti, K.M., J.K. Hatt, E. Petrovskis, and F.E. Löffler.** 2010. Groundwater sampling for nucleic acid biomarker analysis, p. p. 3407-3418. In K.N. Timmis (Ed.), Handbook of Hydrocarbon and Lipid Microbiology. Springer, Berlin.

Student theses

Edwards group

1. **Islam, M.A.** Expected 2013. A systems-level investigation of the metabolism of *Dehalococcoides mccartyi* and the associated community Doctoral thesis, Dept. of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, ON, Canada.

2. **Tang, S.** Expected 2013. Characterization of *Dehalobacter*-containing cultures that reductively dechlorinate chlorinated ethanes and chloroform. Doctoral thesis, Dept. of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, ON, Canada.

3. **Wei, K.** 2012. Substrates and substrate interactions in anaerobic dechlorinating cultures. Master's thesis, Dept. of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, ON, Canada.

4. **Hug, L.A.** 2012. A metagenome-based examination of dechlorinating enrichment cultures: *Dehalococcoides* and the role of non-dechlorinating microorganisms.

Doctoral thesis, Dept. of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, ON, Canada.

5. **Manchester, M.J**. 2011. Characterization of the dechlorinating populations in the WBC-2 consortium. Master's thesis, Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, ON, Canada.

6. **Chan, W.W.M.** 2010. Characterization of reductive dehalogenases in a chlorinated ethene-degrading bioaugmentation culture. Master's thesis, Dept. of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, ON, Canada.

7. **Waller, A.S.** 2009. Molecular investigation of chloroethene reductive dehalogenation by the mixed microbial community KB-1. Doctoral thesis, Dept. of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, ON, Canada.

Löffler group

1. **Padilla-Crespo, E.** Expected 2013. Novel Biomarkers to explore *Dehalococcoides* ecophysiology and support bioremediation applications. Doctoral thesis, Department of Microbiology, University of Tennessee, Knoxville. TN, USA.

2. **Justicia-Leon, S.D.** 2012. Microbes and monitoring tools for anaerobic chlorinated methane bioremediation. Doctoral thesis, School of Biology, Georgia Institute of Technology, Atlanta, GA, USA

3. **Wagner, D.D.** 2012. Comparative genomics reveal ecophysiological adaptations of organohalide-respiring bacteria. Doctoral thesis, School of Biology, Georgia Institute of Technology, Atlanta, GA, USA.

4. **Fletcher, K.E.** 2010. New insights into reductive detoxification of chlorinated solvents and radionuclides. Doctoral thesis, School of Civil and Environmental Engineering, Georgia Institute of Technology, Atlanta, GA, USA.

5. **Thorp, A.** 2009. Identification of reductive dehalogenase genes in *Anaeromyxobacter dehalogenans.* Masters Thesis, School of Biology, Georgia Institute of Techology, Atlanta, GA, USA.

6. **Amos, B.K.** 2007. Evaluation of microbial reductive dechlorination in tetrachloroethene dense nonaqueous phase liquid (DNAPL) source zones. Doctoral thesis, School of Civil and Environmental Engineering, Georgia Institute of Technology, Atlanta, GA, USA.

Oral and Platform Presentations

2013

1. **Löffler, F.E.** 2013. Towards knowledge-based bioremediation: monitoring tools for keystone bacteria. Second International Symposium on Bioremediation and Sustainable Environmental Technologies, June 10-13, 2013. Jacksonville, FL, USA.

2. **Padilla-Crespo E., B. Simsir, K.M. Ritalahti, and F.E. Loeffler.** 2013. Cultures and tools to initiate and monitor 1,2-dichloropropane detoxification at contaminated sites. *Second International Symposium on Bioremediation and Sustainable Environmental Technologies.* June 10–13, 2013. Jacksonville, Florida, USA.

3. **Löffler, F. E.** 05/31/2013. *Dehalococcoides* and knowledge-driven bioremediation. *China-US Workshop on Advances in Environmental Microbiology and Biotechnology.* Nanjing University, May 31, 2013. Nanjing, China.

4. **Edwards, E.A.** 2013. Reductive dehalogenases: intriguing enzymes at the heart of successful chlorinated solvent bioremediation. Arizona State University, March 22, 2013. Phoenix, AZ, USA.

5. **Ritalahti, K.M.** 2013. Incorporating quantitative polymerase chain reaction (qPCR) analyses into site management. *Microbial Insights Webinar Series.* Mar. 15, 2013, Rockford, TN, USA.

6. **Ritalahti, K.M. and C. Jacob.** 2013. In situ bioremediation: trichloropropane and dichloropropane. *2014 Bench Strength - a CPS short Course.* May 6-8, 2013. Loveland, CO, USA.

7. **Ritalahti, K.M.** 2013. Incorporating quantitative polymerase chain reaction (qPCR) analyses into site management. *Microbial Insights Webinar Series.* March 15, 2013, Rockford, TN, USA.

8. **Edwards, E.A.** 2013. Genomics and chlorinated solvent bioremediation. *Bangor University*, February 11, 2013. Bangor, UK.

9. **Löffler, F.E.** 2013. Microbial degradation of chlorinated pollutants: principles and applications. *Microbial Insights Webinar Series*. February 11, 2013, Rockford, TN, USA.

10. **Edwards, E.A.** 2013. How genomic technologies are helping clean up pollutants in groundwater. *Oxford University Structural Genomics Consortium*, February 6, 2013. Oxford, UK.

2012

11. **Löffler, F.E.** 2012. From the lab to the field: enhanced bioremediation of chlorinated solvents. BACSIN, European Union FP7 Framework Program "Bacterial solutions to a cleaner environment", *Trippenhuis, Dutch Royal Institute of Sciences,* March 29-30, 2012. Amsterdam, Netherlands.

12. **Löffler, F.E.** Towards knowledge‐ based bioremediation: monitoring tools for keystone bacteria. *European Union Marie Curie GOODWATER Initial Training Network,* Institute of Groundwater Ecology, Helmholtz Zentrum Munich, Germany.

13. **Edwards, E.A.** 2012. Ascribing function to dehalogenase genes, *Society for Industrial Microbiology and Biotechnology Annual Meeting*. Washington, DC, USA

14. **Edwards, E.A.** 2012. New frontiers in bioremediation, *Eawag*, Dübendorf, Switzerland.

15. **Edwards, E.A., A. Perez de Mora, and L. Lomheim.** 2012. Dehalogenases in the environment and monitoring bioremediation. *Innovative Combined Treatment Technologies for Remediating Contaminated Groundwater Kickoff Meeting.* Toronto, ON, Canada

16. **Ritalahti, K.M., J.K. Hatt, D.M. Ogles, G.A. Davis, B.R. Baldwin and F.E. Löffler**. 2012. Taking the blinders off: The added value of multiple functional gene targets in *Dehalococcoides*-targeted qPCR analysis. *In Abstracts from the 8th International Conference on Remediation of Chlorinated and Recalcitrant Compounds.* May 21-24, 2012. Monterey, CA, USA.

17. **Edwards, E.A.** 2012. Woods Hole course on microbial diversity. *Woods Hole Institute*, July 2012. MA, USA

18. **Zila, A., A. Perez-de-Mora, M. McMaster and E.A. Edwards.** 2012. Tracking microbial populations during enhanced *in situ* bioremediation of a fractured bedrock site contaminated with chloroethenes. *In Abstracts from the 8th International Conference on Remediation of Chlorinated and Recalcitrant Compounds.* May 21-24, 2012. Monterey, CA, USA.

19. **Edwards, E.A.** 2012. Nature to the rescue: new discoveries in detoxifying groundwater contaminants. November 1, 2012. *Public lecture at Trent University*, ON, Canada.

20. **Edwards, E.A.** 2012. New frontiers in bioremediation. *Genomics: the Power and the Promis*e, November 27, 2012. Ottawa, ON, Canada,

2011

21. **Löffler, F.E.** 2011. The value of environmental molecular diagnostics for knowledge-based bioremediation (Keynote Lecture). *SERDP/ESTCP Partners in Environmental Technology Technical Symposium and Workshop,* November 29-December 1, 2011. Washington DC, USA.

22. **Braissant, O., S. Spring, C. Lebron, K.M. Ritalahti, F.E. Löffler and N.M. Lee.** 2011. Molecular biological tools and microcalorimetry: a novel approach to estimate cellular activities. *REMTEC Remediation Technology Summit - The Future of Remediation Technology*, May 16-19 2011. Chicago, IL, USA.

23. **Edwards, E.A**. 2011. The benefits of microbial genomics to the environment. *Ontario Genomics Institute.* ON, Canada.

24. **Edwards, E.A**. 2011. New frontiers in bioremediation. *61st Canadian Chemical Engineering Conference*. London, ON, Canada.

25. **Islam, M.A., E.A. Edwards, and R. Mahadevan.** 2011. Investigating the metabolism of *Dehalococcoides* and associated community members. *Genome Biology and Bioinformatics Work in Progress seminar series*. Toronto, ON, Canada.

26. **Lee, N. M., O. Braissant, S. Spring, J. Reitner, W. Liebl and F.E. Löffler.** 2011. Capturing and visualizing the activity and biodiversity of microbes in the subsurface. *8th International Subsurface Symposium*. September 11-16, 2011, Garmisch-Partenkirchen, Germany.

27. **Ritalahti, K., J. Hatt, F. Löffler, N. Barros, C.Crea, D. Major, E. Petrovskis, G. Davis, D. Ogles, P. Dennis, X. Druar, J. Wilkinson, M. Duhamel, E. Edwards and A. Perez de Mora, C. Yeager and C. LeBrón.** 2011. Accurate enumeration of *Dehalococcoides* biomarker genes in groundwater. *Battelle International Symposium on Bioremediation and Sustainable Environmental Technologies,* June 27-30, 2011. Reno, NV, USA.

28. **Edwards, E.A.** 2011. Session on identifying and modeling biodegradative pathways. *Battelle International Symposium on Bioremediation and Sustainable Technologies*. June 27-30, 2011. Reno, NV, USA.

29. **Manchester, M., M. Zarek, L. Hug, E.A. Edwards, S. Dworatzek, and M. Lorah.** 2011. Characterization of dechlorinating populations in the WBC-2 consortium. *In Abstracts from the International Symposium on Bioremediation and Sustainable Environmental Technologies,* June 27-30, 2011. Reno, NV, USA.

30. **Tang, S., A. Grostern, W. Chan, and E.A. Edwards.** 2011. *Dehalobacter* unveiled: A key player in the detoxification of chlorinated alkanes at contaminated sites. *In Abstracts from the International Symposium on Bioremediation and Sustainable Environmental Technologies,* June 27-30, 2011. Reno, NV, USA.

31. **Wei, K., S. Tang, and E.A. Edwards.** 2011. Biodegradation of chlorofluorocarbon-113 in anaerobic enrichment cultures. *13th CSChE Ontario-Quebec Biotechnology Meeting.* Kingston, ON. Canada.

32. **Ritalahti, K.** 2011. Essential community interactions supporting organohalide respiration by *Dehalococcoides*. *NIMBioS Investigative Workshopon Individual-Based Ecology of Microbes: Obervations and modeling.* June 8-10, 2011. Knoxville, TN, USA.

33. **Löffler, F. E.** 2011. Ecophysiology of organohalide-respiring bacteria. Web-based lecture to biology undergraduate students. University of Puerto Rico, Mayagüez. August 2011. Mayagüez, PR.

2010

34. **Löffler, F. E.** 2010. Bioremediation: Science-based engineering of the contaminated subsurface. State Environmental Protection Key Laboratory of Environmental Risk Assessment and Control on Chemical Process, School of Resource and Environmental Engineering, East China University of Science and Technology (ECUST), October 23, 2010. Shanghai, China.

35. **Löffler, F.E.** 2010. Bioremediation: Science-driven engineering of the contaminated Subsurface. *EHPC 2010 - International Workshop on Environmental Health & Pollution Control*, October 16-23 2010, Nanjing University, Nanjing, and China Normal University, Beijing, China.

36. **Löffler, F. E.** 2010. Novel insights into the physiology of organohalide-respiring bacteria. Symposium on Advances in Microbial Ecophysiology and Biochemistry, *Max Planck Institute for Marine Microbiology*, October 9, 2010. Bremen, Germany.

37. **Löffler, F.E.** 2010. Ecology of organohalide-respiring *Dehalococcoides*. 13th *International Symposium on Microbial Ecology.* August 22-27, 2010, Seattle, WA, USA.

38. **Braissaint, O, K. I. Jönsson, R. Aubrecht, L. Kovavic, C. Heim, N.V. Queric, V. Thiel, J. Reitnjer, S. Spring, K.M. Ritalahti, W. Liebl, F.E. Löffler and N.M. Lee.** 2010. Isothermal microcalorimetry: a non-disruptive tool for analysis of microbial heat production for evaluation of in situ activities and cell numbers. *ISME 13 International Symposium on Microbial Ecology Microbes*. August 22-27, 2010. Seattle, WA. USA.

39. **Ritalahti, K., J Hatt, F. Loeffler, N. Barros, D. Major, E. Ney, G. Davis, D. Ogles, P. Dennis, J. Wilkinson, W. Chan, M. Duhamel, E. Edwards, H. Vrionis, C. LeBrón anc C. Yeager.** 2010. Use of microbial internal controls for assessment of *Dehalococcoides* biomarker genes in groundwater. *In Abstracts from the Seventh*

International Conference on Remediation of Chlorinated and Recalcitrant Compounds, May 24-27, 2010. Monterey, CA, USA.

40. **Löffler, F.E.** 2010. Introduction to molecular biological tools (MBTs). *Interstate Technology & Regulatory Council (ITRC) environmental molecular diagnostics team meeting,* April 19-20, 2010, Austin, TX, USA.

41. **Löffler, F.E.** 2010. Exploiting nature's blueprint in bioremediation. *The University of Tennessee,* February 2010. Knoxville, TN. USA.

42. **Edwards, E.A.** 2010. Metagenomics and bioremediation: from genomes to solutions. University of Toronto, Scarborough Campus.

43. **Edwards, E.A.** 2010. Metagenomics and bioremediation: from genomes to solutions. Ryerson University, Toronto, ON, Canada.

44. **Edwards, E.A**. 2010. Metagenomics and bioremediation: from genomes to solutions. *Functional Genomics: Present and Future Symposium.* University Health Network Microarray Centre, Toronto, ON, Canada.

45. **Edwards, E.A**. 2010. New frontiers in bioremediation. Centre for Environment, University of Toronto, Toronto, ON, Canada.

46. **Edwards, E.A**. 2010. Bioremediation of halogenated organic compounds in soils and groundwater. *Cool Climate Oenology & Viticulture Institute*. St. Catharines, ON, Canada.

47. **Edwards, E.A**. 2010. New frontiers in Bioremediation. *Canadian Society for Microbiology Conference*. Hamilton, ON, Canada.

48. **Edwards, E.A**. 2010. New frontiers in bioremediation. *TEDxToronto*. Toronto, ON, Canada.

49. **Hug, L.A., and E.A. Edwards**. 2010. Bioremediation of chlorinated solvents in the lab vs. in the field: the complexity paradox. *EnviroAnalysis*. Toronto, ON, Canada

50. **Islam, M.A., E.A. Edwards, and R. Mahadevan.** 2010. Elucidating metabolic bottlenecks and visualizing gene-expression data with a pan-genome-scale metabolic model of *Dehalococcoides.Genome Biology and Bioinformatics Work in Progress Seminar Series*.University of Toronto, Toronto, ON, Canada

51. **Islam, M.A., E.A. Edwards, and R. Mahadevan.** 2010. Pan-genome-scale modeling of *Dehalococcoides* metabolism. *12th Annual Ontario-Quebec CSChE Biotechnology Meeting*. Montreal, QC, Canada.

52. **Meisinger, D.B., E. Padilla-Crespo, K.M. Ritalahti, H. Schmidt, M. Schmid, C. Lebrón, F.E. Löffler and N.M. Lee.** 2010. An inventory of the global microbial diversity of chloroorganic contaminated aquifers. *Annual VAAM meeting*, March 28-31, 2010. Hannover, Germany.

53. **Meisinger, D.B., E. Padilla-Crespo, K.M. Ritalahti, S. Spring, H. Schmidt, M. Schmid, C. Lebrón, F.E. Löffler, and N.M. Lee**. 2010. Screening of known and novel reductively dechlorinating species within *Chloroflex*i. *7th International conference remediation of chlorinated and recalcitrant compounds.* May 24-27, 2010. Monterey, CA, USA.

2009

54. **Löffler, F.E.** 2009. Nucleic acid-based quantification of *Dehalococcoides* in groundwater. Partners in Environmental Technology, Technical Symposium & Workshop, Dec. 1-3, Washington, DC, USA.

55. **Löffler, F. E.** 2009. Levels of complexity in a simple system - Organohalide respiration by *Dehalococcoides*. Microbes to Metazoans: Regulation, Dynamics, and Evolution of Social Behavior. Dec. 2-4, 2009, Georgia Institute of Technology, Atlanta, GA, USA.

56. **Ritalahti, K. M.** 2009. Insights to *Dehalococcoides* analysis and interpretation for bioremediation: Opportunities and Challenges Ahead. *In* Abstracts from the 25th Annual International Conference on Soils, Sediments, Water and Energy, Oct 19-22, 2009. Amherst, MA, USA.

57. **Duhamel, M., and E.A. Edwards**. 2009. Biotechnology for contaminated site remediation. *Pollutec*. Paris, France.

58. **Edwards, E.A., L.A. Hug, and C.E. Devine.** 2009. Metagenome sequences of two industrially relevant mixed microbial consortia. Joint Genomes Institute group meeting with members of the JGI sequencing facility, and representatives from the Spormann, Löffler, Richardson, and Alvarez-Cohen lab groups. JGI, June 3, 2009. Walnut Creek, CA, USA.

59. **Edwards, E.A.** 2009. Bioaugmentation for **C**hlorinated Solvent Remediation. Department of Civil and Environmental Engineering and Bio-X program. May date? 2009, Stanford University, Palo Alto, CA, USA.

60. Edwards, E.A. 2009. Bioaugmentation - What's Next? *Tenth International In Situ and On-Site Bioremediation Symposium.* May 5-8, 2009, Baltimore, MD, USA.

61. **Edwards, E.A.** 2009. Harnessing dehalogenating bacteria for environmental cleanup. *109th ASM General Meetin*g. May 17-21, Philadelphia, PA, USA.

62. **Edwards, E.A**. 2009. CSIA to distinguish metabolic pathways in microbial systems. *Remediation Technology Summit (RemTEC09).* March 3-5, 2009, Atlanta, GA, USA.

63. **Fletcher, K.E., I. Nijenhuis, H. -H. Richnow, and F.E. Löffler.** 2009. Stable carbon isotope fractionation of 1,2-dichloropropane during degradation by

Dehalococcoides populations. *Remediation Technology Summit (RemTEC09).* March 3-5, 2009, Atlanta, GA, USA.

64. **Hauer, K., D.B. Meisinger, M. Pavlekovic, A.K. Kniggendorf, S.H. Thomas; J. Chee-Sanford, R. Sanford, C. Lebrón, W. Liebl, F.E. Löffler and N.M. Lee.** 2009. Novel tools for in situ detection of biodiversity and function of dechlorinating and uranium-reducing bacteria in contaminated environments. *Goldschmidt Meeting: Challenges to our volatile planet.* June 21-26, 2009, Davos, Switzerland.

65. **Hug, L.A.** 2009. Chemical Pollution and Bioremediation: Systems biology approaches for understanding microbial solutions to contamination. *Cell and Systems Biology student seminar series.* March 11, 2009 University of Toronto, Toronto, ON, Canada.

66. **Lee, N. M., D. B. Meisinger, S. Spring, G. Diekert, H. H. Richnow, C. A. Lebrón, and F.E. Löffler.** 2009. Visualizing microorganisms by fluorescence in situ hybridization in contaminated environments - potentials and limits. *Remediation Technology Summit (RemTEC09),* March 3-5, 2009, Atlanta, GA, USA.

67. **Lee, N. M., D.B. Meisinger, S. Spring, G. Diekert, H.H. Richnow, C. Lebrón, and F.E. Löffler.** 2009. Visualizing microorganisms by fluorescence in situ hybridization in contaminated environments – potentials and limits. *REMTEC9 Remediation Technology Summit: The Future of Remediation Technology.* March 2-5, 2009, Atlanta, GA, USA.

68. **Lee, N.M., D.B. Meisinger, E. Padilla-Crespo, S. Spring, A.S. Engel, M. Schmid, K.M. Ritalahti and F.E. Löffler.** 2009. Exploring the global evolution, biodiversity and past and current roles of the phylum *Chloroflexi* - one of the poorly defined, deep-branching phyla in the domain of Bacteria. *CAREX workshop Life in Extreme Environments Projects.* October 28-30, 2009, Prague (Academy of Sciences of the Czech Republic), Czech Republic.

69. **Löffler, F.E.** Towards environmental systems biology of *Dehalococcoides* for enhanced bioremediation. 2009. *Gordon Conference on Applied & Environmental Microbiology: from single cells to the environment.* July 12-17, 2009, Mount Holyoke College, South Hadley, MA, USA

70. **Löffler, F.E.** 2009. Bioremediation and *Dehalococcoides.* Stanford's Hopkins Marine Station. June 2009, Pacific Grove, CA, USA

71. **Löffler, F.E.** 2009. Bioremediation: Science-driven engineering of the contaminated subsurface. Department of Civil and Environmental Engineering and Bio-X program, Stanford University. May 2009, Palo Alto, CA, USA.

72. **Löffler, F.E.** 2009. *Dehalococcoides* and bioremediation of chlorinated solvents. Casa Pueblo, ,October 2009. Adjuntas, PR.

73. **Löffler, F.E.** 2009. Environmental microbiology: from genomes to solutions. Bioinformatics and Genomics Joint Seminar Series, College of Computing, College of Science and College of Engineering, Georgia Institute of Technology, February 2009.Atlanta, GA, USA.

74. **Padilla-Crespo, E., C. Cruz-García, K.M. Ritalahti, C. Lebrón, L. Hung, A.S. Waller, E.A. Edwards, F.E. Löffler.** 2009. Application of an oligonucleotide microarray for reductive dechlorination biomarker identification and process monitoring. *Remediation Technology Summit (RemTEC09)*. March 3-5, 2009, Atlanta, GA, USA.

75. **Padilla-Crespo, E., C. Cruz-García, K.M. Ritalahti, C. Lebrón, L. Hung, A.S. Waller, E.A. Edwards, F.E. Löffler.** 2009. Design and application of an oligonucleotide microarray for monitoring reductive dechlorination processes. *Battelle In-Situ and On-Site Bioremediation Symposium*. May 5-8, 2009, Baltimore, MD, USA.

76. **Ritalahti, K.M., B.K. Amos, K.E. Fletcher, E. Padilla-Crespo, C. Cruz-Garcia, N.L. Cápiro, J. Costanza, K.D. Pennell, and F.E. Löffler.** 2009. Bioaugmentation with *Dehalococcoides* is a bioremediation success story. *In Situ and On-Site Bioremediation Symposium*. May 5-8, 2009, Baltimore, MD, USA.

77. **Ritalahti, K.M.** 2009. Molecular tools for microbial reductive dechlorination assessment. *19th Annual Training Conference of the National Association of Remedial Project Managers (NARPM).* June 5, 2009, Atlanta, GA, USA.

78. **Ritalahti, K.M., J.K. Hatt, E. Petrovskis, K. Henn, C. Lebrón and F.E. Löffler. 2009**. Standardization and comparison of sampling and DNA extraction procedures for analysis of biomarkers in groundwater. *Remediation Technology Summit (RemTEC09).* March 3-5, 2009, Atlanta, GA, USA.

79. **Waller, A.S., L. Hug, M. Duhamel, A. Islam, R. Mahadevan and E.A. Edwards.** 2009. Characterizing *Dehalococcoides* physiology using genomics and transcriptomics. *Society for Industrial Microbiology*. July 2009, Toronto, ON, Canada.

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80. **Edwards, E.A.** 2008. Bioremediation: genomes to solutions. Genome Biology and Bioinformatics Retreat. May 14, 2008, Toronto, ON, Canada.

81. **Edwards, E.A.** 2008. Molecular biology tools in bioremediation. Air Force Center for Environment and Engineering (AFCEE) workshop, March 2008, San Antonio, TX, USA.

82. **Lee, N.M., R. Rossello-Morá, H.J. Busse, R. Geyer, C.M. L. Larsson, W. Ludwig, M. Schmid, B. Szponar, B. Tindall, I. Tjernberg, F.E. Löffler, H. Reichenbach, and K.-H. Schleifer**. 2008. Screening, enrichment and isolation of novel members within the phylum *Chloroflexi* from the environment. *IUMS conferences XII International Congress of Bacteriology and Applied Microbiology*. August, 5-15, 2008, Istanbul, Turkey.

83. **Lee, N.M., H. J. Busse, A.S Engel, G. M., Garrity, C.M. Henk, D.B. Meisinger, M. Pavlekovic, R. Rossello-Morá, S. Spring, B. Tindall, and F.E. Löffler.** 2008. Novel insights into the biogeography and phylogeny of the phylum *Chloroflexi*. *ISME 12 - Microbial Ecology*. August 17-22, 2008. Cairns, Australia.

84. **Lee, N. M., D.B. Meisinger, M. Pavlekovic, K. Hauer, A.S. Engel, G.M. Garrity, W. Ludwig, K. H. Schleifer, and F.E. Löffler**. 2008. The biodiversity of *Chloroflexi*-affiliated bacteria in the subsurface. *7th ISSM Symposium of Subsurface Microbiology: The Challenge of the Subsurface the Fourth Environment for Life*. November 16-21, 2008, Shizuoka, Japan.

85. **Löffler F.E., K.M. Ritalahti, E.A. Edwards, N.M. Lee and C. Lebrón**. 2008. BioReD: Biomarkers and tools for reductive dechlorination site assessment, monitoring, and management. *6th International Conference on Remediation of Chlorinated and Recalcitrant Compounds.* May 19-22, 2008, Monterey, CA, USA.

86. **Löffler, F.E.** 2008. "New age" bioremediation: From reductionism to environmental systems biology. October 2008, College of Engineering, Tufts University, Boston, MA, USA.

87. **Löffler, F.E.** 2008. Novel insights into the microbial reductive dechlorination process. *Annual Meeting and International Symposium of the Ecological Society of China.* November 2008, Central South University, Changsha, Hunan Province, China.

88. **Löffler, F.E.** 2008. Towards Environmental Systems Biology of *Dehalococcoides* for Enhanced Bioremediation. *Integrative BioSystems Institute Launch Conference*. October 18-21, 2008, Atlanta, GA, USA.

89. **Löffler, F.E**. 2008. Bioremediation: Science-driven engineering of the contaminated subsurface. August 2008, Oak Ridge National Laboratory, Oak Ridge, TN, USA.

90. **Löffler, F.E**. 2008. Bioremediation: science-driven engineering of the contaminated subsurface. Department of Civil and Environmental Engineering and Department of Microbiology, University of Tennessee, August 2008, Knoxville, TN, USA.

91. **Löffler, F.E.** 2008. Microbial processes in the hyporheic zone that affect the fate of pollutants. DFG Round-Table Workshop. *Interactions of chemical, physical, and microbiological processes in the hyporheic zone and their effect on xenobiotic compounds.* August 2008. Nuremberg, Germany

92. **Löffler, F.E., K.M. Ritalahti, E.A. Edwards, N.M. Lee, and C. Lebrón.** 2008. BioReD: Biomarkers and tools for reductive dechlorination site assessment, monitoring, and management. *Battelle 6th International Conference on Remediation of Chlorinated and Recalcitrant Compounds.* May 19-22, 2008, Monterey, CA, USA.

93. **Löffler, F.E.** 2008. Exploiting nature's blueprint in biotechnology. Department of Microbiology and Center for Biocatalysis and Bioprocessing. , University of Iowa, April 30-May 1, 2008. Iowa City, IA, USA.

94. **Löffler, F.E.** 2008. Exploiting nature's blueprint for bioremediation. Department of Civil and Environmental Engineering, Michigan State University, April 10, 2008. East Lansing, MI, USA

95. **Ritalahti, K.M., S.H. Thomas, A. Nevins, and F.E. Löffler.** 2008. Advanced quantitative PCR techniques for groundwater microbial monitoring. *Battelle 6th International Conference on Remediation of Chlorinated and Recalcitrant Compounds.* May 19-22, 2008, Monterey, CA, USA.

96. **Meisinger, D.B., M. Pavlekovic, K.U. Totsche, V. Radl, M. Schloter, M. Schmid, C. Lebrón, K.M. Ritalahti, F.E. Löffler and N.M. Lee**. 2008. Characterization of the microbial community in a TCE-contaminated landfill in Bavaria, Germany. *Annual Annual VAAM meeting*. March 9-11 2008, Frankfurt, Germany.

97. **Meisinger D.B., K. Hauer, S. Hao, S., Spring, I. Nijenhuis, H.H. Richnow, C. Lebrón, F.E. Löffler and N.M. Lee.** 2008. Visualizing reductively dechlorinating bacteria in environmental samples using fluorescence in situ hybridization - potential and limits. *EC-RSSG-1*. October, 21-23, 2008, Amsterdam, The Netherlands.

98. **Meisinger, D. B., M. Pavlekovic, K.U. Totsche, V. Radl, M. Schloter, M. Schmid, K.M. Ritalahti, C. Lebrón, F.E. Löffler and N.M. Lee**. 2008. Systematic comparison of different molecular tools for characterization of a TCE-contaminated aquifer. *EC-RSSG-1*. October 21-23 2008, Amsterdam, The Netherlands.

99. **Meisinger, D.B., M. Pavlekovic K.U. Totsche, V. Radl, M. Schloter, M. Schmid, K.M. Ritalahti, C. Lebrón, F.E. Löffler and N. M. Lee**. 2008. Systematic evaluation of current molecular tools for characterizing the microbial diversity of chlorinated solvent contaminated aquifers. *ISSM Symposium of Subsurface Microbiology: The Challenge of the Subsurface - the Fourth Environment for Life*. November 16-21, 2008, Shizuoka, Japan.

100. **Pavlekovic, M., K. Hauer, S. Hao, S. Spring, I. Nijenhuis, H. H. Richnow, C.M. Henk, M. Fiandaca, C. Lebrón, C., S.H. Thomas, K.M. Ritalahti, F.E. Löffler and N.M. Lee.** 2008. Development and optimization of in situ detection techniques for reductively dechlorinating bacteria in TCE-contaminated groundwater systems. *Virtual Institute for Isotope Biogeochemistry Workshop: Electron transfer processes at biogeochemical gradients.* March 3-7, 2008, Leipzig, Germany.

2007

101. **Waller, A.S., and Edwards, E.A.** 2007. The use of microarrays to elucidate biomarkers of reductive dechlorination. *2nd International Conference on Challenges in Site Remediation,* September, 2007. Niagara Falls, NY, USA.

102. **Edwards, E.A.** 2007. Bioremediation: from genomes to solutions". EuroBio, September 25-28, 2007 Lille, France.

103. **Edwards, E.A.** 2007. Bioremediation: from genomes to solutions" Society for Industrial Microbiology, August 2007. Denver CO, USA.

104. **Löffler, F. E.** 2007. Bioremediation - Science-driven engineering of the contaminated subsurface. Department of Microbiology, University of Massachusetts, Amherst, MA, July, 2007.

105. **Löffler, F. E.** 2007. From the plume to the source: What microbes can do in DNAPL source zones. Interstate Technology & Regulatory Council (ITRC) BioDNAPL Team Meeting, June 13th-14th, 2007, Las Vegas, NV.

106. **Löffler, F. E.** 2007. New microbes - A blueprint for innovative chlorinated solvent bioremediation. DuPont Corporate Remediation Group, February 6, 2007, Newark, DE.

107. **Löffler, F. E.** 2007. Molecular biological tools (MBTs) to support efficient *in-situ* Bioremediation. Florida Department of Environmental Protection (FDEP) Contractor Workshop, Tallahassee, FL, February 28-March 1, 2007.

108. **Löffler, F. E.** 2007. Anaerobic microbial detoxification of chlorinated pollutants. Department of Civil and Environmental Engineering, Yonsei University, Seoul, Republic of Korea, February 2007.

109. **Löffler, F. E.** 2007. Chlorinated solvent bioremediation. Korea Research Institute of Bioscience & Biotechnology, February 8, Daejon, Republic of Korea, February 2007.

Poster Presentations

2013

1. **Koenigsberg, S., M. Kram, R. Harwood, F. Lakhwala, F. Löffler, D. Ogles, R. Pirkle, G. Gustafson, and S. Britt.** 2013. A multivariate diagnostic strategy to examine product performance claims. *Second International Symposium on Bioremediation and Sustainable Environmental Technologies*. June 10-13, 2013, Jacksonville, FL, USA.

2. **Padilla-Crespo, E., B. Simsir, K. M. Ritalahti, and F. Löffler.** 2013. Cultures and tools to initiate and monitor of 1,2-dichloropropane detoxification at contaminated sites. *Second International Symposium on Bioremediation and Sustainable Environmental Technologies*. June 10-13, 2013, Jacksonville, FL, USA.

3. **Padilla-Crespo E., J. Yan, C. Swift, K. Chourey, K.M. Ritalahti, R.L. Hettich, and F.E. Loeffler.** 2013. Identification and environmental distribution of the 1,2-dichloropropane-to-propene reductive dehalogenase *dcpA*. *Seventh International Conference on Remediation of Contaminated Sediments*. February 4-7, 2013, Dallas, TX, USA.

2012

4. **Hug, L.A., P.J. McMurdie, P.C. Blainey, A. Sczyrba, T. Woyke, S.R. Quake, A.M. Spormann, and E.A. Edwards.** 2012. Single cell amplified genomes of three dechlorinating organisms reveal mechanisms of co-operative reductive dechlorination of chlorinated solvents. *112th General Meeting of the American Society for Microbiology*. San Francisco, CA, USA.

5. **Islam, M.A., E.A. Edwards, and R. Mahadevan.** 2012. Investigating *Dehalococcoides* transcriptomes with a pan-genome-scale metabolic model. *Genome Biology and Bioinformatics Annual Retreat*. Toronto, ON, Canada.

6. **Lee, N. M., C. Lébron, E. Padilla-Crespo, J. Yan, K.M. Ritalahti and F.E. Löffler.** 2012. Novel Molecular Biological Tools to Visualize Bacteria Involved in Reductive Dechlorination. *8th International Conference on Remediation of Chlorinated and Recalcitrant Compounds*. May 21-24, 2012, Monterey, CA, USA.

7. **Molenda, O., X. Liang, L. Hug, M. Manchester, S. Tang and E.A. Edwards.** 2012.Deducing the role of reductive dehalogenase enzymes in mixed microbial cultures used for bioremediation of 1,1,2,2-tetrachloroethane. *International Society for Microbial Ecology Symposium (ISME14)*. Aug. 19-24, 2012, Copenhagen, Denmark.

8. **Padilla-Crespo, E., J. Yan, F.E. Löffler.** 2012. Use of nucleic acid-based tools to predict and monitor detoxification of 1,2-dichloropropane at contaminated sites. 2012

American Society of Microbiology General Meeting. June 16-19, 2012, in San Francisco, CA, USA.

9. **Padilla-Crespo, E., K.M. Ritalahti, and F.E. Löffler.** 2012. Identification of reductive dehalogenase genes for enhanced bioremediation tools and *Dehalococcoides* monitoring. *Eighth International Conference on Remediation of Chlorinated and Recalcitrant Compounds*. May 21–24, 2012, Monterey, CA, USA.

10. **Wei, K. and E.A. Edwards.** 2012. Co-contaminant effects and cross-inhibition in dechlorinating enrichment cultures. *INTEGRATE 1st Annual Meeting*, September 28, 2012. Toronto, ON, Canada.

11. **Wei, K. and E.A. Edwards.** 2012. Co-contaminant effects and cross-inhibition in dechlorinating enrichment cultures. *International Society for Microbial Ecology Symposium (ISME14).* Aug. 19-24, 2012, Copenhagen, Denmark.

2011

12. **Hug, L.A., P.J. McMurdie, A.S. Waller, S. Holmes, A. Spormann, and E.A. Edwards.** 2011. Testing an enrichment culture metagenome binning heuristic with a dechlorinating microbial consortium. *Genomics of Energy & Environment: 6th Annual DOE JGI User Meeting*. Walnut Creek, CA, USA.

13. **Hug, L.A., A.R. Rowe, D. Parks, R.G. Beiko, R.E. Richardson, and E.A. Edwards.** 2011. Comparative metagenomics of three *Dehalococcoides*-containing dechlorinating microbial consortia. *American Society for Microbiology General Meeting*. New Orleans, LA, USA.

14. **Islam, M.A., E.A. Edwards, and R. Mahadevan.** 2011. New insights into *Dehalococcoides* metabolism from an integrated metabolic transcriptomics study. *Biochemical and Molecular Engineering XVII Conference*. Seattle, WA, USA.

15. **Islam, M.A., E.A. Edwards, and R. Mahadevan.** 2011. A systems-level study of the transcriptional changes of *Dehalococcoides* metabolism. *Gordon Research Conference: Cellular Systems Biology*. Davidson, NC, USA.

16. **Lee, N.M., C. Lebrón, K.M. Ritalahti, and F.E. Löffler.** 2011. An integrated molecular biological approach to visualize the biodiversity and ecology in chloro-organic contaminated systems. *Partners in Environmental Technology Technical Symposium and Workshop.* 29 November - 1 December 2011, Washington DC, USA.

17. **Padilla-Crespo, E., J. Yan, K.M. Ritalahti, F.E. Löffler, S. Tang, and E.A Edwards.** 2011. Identification of reductive dechlorination biomarker genes for chlorinated solvent site assessment and bioremediation monitoring. *Partners in Environmental Technology Technical Symposium & Workshop*. November 29-December 1, 2011, Washington, DC, USA.

18. **Padilla-Crespo E., K.M. Ritalahti, and F.E. Löffler.** Identification of a 1,2-dichloropropane reductive dehalogenase gene and evidence of horizontal gene transfer between the *Chloroflexi. 8th International Symposium of Subsurface Microbiology*. September 11-16, 2011, Garmisch-Partenkirchen, Germany.

19. **Padilla-Crespo E., and F.E. Löffler**. Ecology and distribution of a new biomarker linked to 1,2-dichloropropane dechlorination in subsurface environments. *American Geophysical Union Fall Meeting*. December 5-9, 2011, San Francisco, CA, USA.

20. **Tang, S., and E.A. Edwards**. 2011. Identification of *Dehalobacter* reductases that dechlorinate 1,1,1-trichloroethane, chloroform and 1,1-dichloroethane. *American Society for Microbiology General Meeting*. New Orleans, LA, USA.

2010

21. **Justicia-Leon, S.D., S. Tang, A. Grostern, M. Duhamel, S. Dworatzek, P. Dennis, E.A. Edwards, and F.E. Löffler.** 2010. Bioaugmentation with a *Dehalobacter*-containing consortium initiates chloroform reductive dechlorination. *Batelle 7th International Conference for Remediation of Chlorinated and Recalcitrant Compounds*. Monterey, CA, USA.

22. **Ritalahti, K., J Hatt, F. Löffler, N. Barros, D. Major, E. Ney, G. Davis, D. Ogles, P. Dennis, J. Wilkinson, W. Chan, M. Duhamel, E. Edwards, H. Vrionis, C. LeBrón and C. Yeager.** 2010. Use of microbial internal controls for assessment of Dehalococcoides biomarker genes in groundwater. *In Abstracts from the Seventh International Conference on Remediation of Chlorinated and Recalcitrant Compounds*, May 24-27, 2010. Monterey, CA, USA.

23. **Fletcher, K.E., L. Adrian, J. Seifert, W. Chan, and F.E. Löffler**. 2010. Identification of a reductive dehalogenase catalyzing hydrogenolysis and dichloroelimination reactions in *Dehalococcoides* sp. strain BAV1. *110th General Meeting of the American Society for Microbiology*. May 23-27, 2010, San Diego, CA, USA.

24. **Justicia-Leon, S. D., E. Padilla-Crespo, J.K. Hatt, K. M. Ritalahti, J. Yan, E.E. Mack, and F.E. Löffler**. 2010. Ecology and distribution of bacteria capable of anaerobic transformation of chlorinated methanes. *13th International Symposium on Microbial Ecology*. August 22-27, 2010, Seattle, WA, USA.

25. **Hug, L.A., P.J. McMurdie, A.S. Waller, S. Holmes, A. Spormann, and E.A. Edwards.** 2010. Testing a novel metagenome binning heuristic with an enrichment culture simulated metagenome. *13th International Symposium for Microbial Ecology*. Seattle, WA, USA.

26. **Islam, M.A., E.A. Edwards, and R. Mahadevan.** 2010. Visualizing gene-expression data with a pan-genome-scale metabolic model of *Dehalococcoides. Genome Biology and Bioinformatics Annual Retreat.* Toronto, ON, Canada

27. **Löffler, F.E., K.M. Ritalahti, E.A. Edwards, and N.M. Lee.** 2010. Comprehensive MBT approaches for site assessment and bioremediation monitoring at chlorinated solvent sites. *Partners in Environmental Technology Technical Symposium and Workshop.* November 30 – December 2. Washington DC, USA.

28. **Lee, N.M., D.B. Meisinger, S. Spring, M. Schmid, M. Rothballer, K. M. Ritalahti, C.A. Lebrón, and F.E. Löffler.** 2010. Screening of known and novel reductively dechlorinating species within Chloroflexi. *International Conference on Remediation of Chlorinated and Recalcitrant Compounds.* May 24-27, 2010, Monterey, CA, USA.

29. **Manchester, M., M. Zarek, S. Dworatzek, M. Lorah, L.A. Hug, and E.A. Edwards.** 2010. Characterization of dechlorinating populations in the WBC-2 consortium. *13th International Symposium on Microbial Ecology*. Seattle, WA, USA.

30. **McMurdie II, P.J., L.A. Hug, F.E. Löffler, E.A. Edwards, and A. Spormann.** 2010. Patterns of horizontal gene transfer in vinyl chloride respiration of *Dehalococcoides* as revealed by comparative (meta) genomics. *110th General Meeting of the American Society for Microbiology*. San Diego, CA, USA

31. **Wagner, D.D., J. Yan, K.M. Ritalahti, K.T. Konstantinidis, and F.E. Löffler**. 2010. Characterization of the *Dehalococcoides c*orrinoid salvage system. 110th General Meeting of the American Society for Microbiology. May 23-27, 2010, San Diego, CA, USA.

32. **Tang, S., A. Grostern, W. Chan, and E.A. Edwards.** 2010. Characterization of *Dehalobacter* cultures that reductively dechlorinate chlorinated ethanes and chloroform. *13th International Symposium on Microbial Ecology*. Seattle, WA, USA.

33. **Yan, J., D.D. Wagner, K.M. Ritalahti, and F.E. Löffler**. 2010. Interspecies cobalamin transfer fulfills "*Dehalococcoides*" growth factor requirement. 110th General Meeting of the American Society for Microbiology. May 23-27, 2010, San Diego, CA, USA.

2009

34. **Chan, W, F.E. Löffler and E.A. Edwards.** 2009. Kinetics of TCE, *cis*-DCE and VC dechlorination by *Dehalococcoides*-containing cultures in the presence of chlorinated ethanes. *Tenth International In Situ and On-Site Bioremediation Symposium.* May 5-8, Baltimore, MD, USA.

35. **Cruz-García, C, J.K. Hatt, K.M. Ritalahti, E. Padilla-Crespo, and F.E. Löffler.** 2009. Optimized procedure for quantitative analysis of reductive dechlorination biomarker transcripts. *Remediation Technology Summit* (RemTEC09). March 3-5, 2009, Atlanta, GA, USA.

36. **Dumas, M. D., K.E. Fletcher, R.R. Helton, K.M. Ritalahti, F.E. Löffler, and K.E. Wommack**. 2009. Dehalophage dynamics in *Dehalococcoides* sp. strain BAV1 cultures. *American Society for Microbiology 109th General Meeting.* May 17-21, 2009, Philadelphia, PA, USA.

37. **Fletcher, K.E., S.H. Thomas, Q. Wu, M.J. Beazley, M.I. Boyanov, K.M. Kemner, and F.E. Löffler**. 2009. Uranium reduction is a common trait of *Desulfitobacterium* spp., *American Society for Microbiology 109th General Meeting.* May 17-21, 2009. Philadelphia, PA, USA.

38. **Hauer, K., M. Pavlekovic, M. Pilhofer, K. Zwirglmaier, W. Ludwig, K.-H. Schleifer, F.E. Löffler, and N.M. Lee.** 2009. In situ detection of low copy genes with RING-FISH (Recognition of Individual Genes): possibilities and limitations. *Gordon research conference: From Single Cells to the Environment.* July 12-17, 2009, South Hadley, MA, USA.

39. **Hug, L.A., P.J. McMurdie, A.S. Waller, S. Holmes, A. Spormann, and E.A. Edwards.** 2009. Testing an enrichment culture metagenome binning heuristic with a dechlorinating microbial consortium. *7th Annual RECOMB Satellite Workshop on Comparative Genomics*. Budapest, Hungary.

40. **Islam, M.A., E.A. Edwards and R. Mahadevan**. 2009. A Pan-genome-scale metabolic model for *Dehalococcoides*. *American Society for Microbiology 109th General Meeting*. May 17-2, 2009, Philadelphia, PA, USA.

41. **Lee, N.M., D.B. Meisinger, K.M. Ritalahti, S. Spring, M. Schmid, and F.E. Löffler.** 2009. Symbiotic based cultivation for improved cultivation strategy of novel species within the phylum *Chloroflexi*. *VAAM workshop on Symbiotic Interaction,* Helmholtz German Research Center for Environmental Health. November 19-20, 2009, Neuherberg/Munich, Germany.

42. **McMurdie, P., F. Löffler, and A. Spormann**. 2009. Elucidating the role of horizontal gene transfer in vinyl chloride respiration of *Dehalococcoides* through comparative genomics. *SERDP/ESTCP Partners in Environmental Technology Technical Symposium & Workshop*. December 1-3, 2009, Washington, DC, USA.

43. **Meisinger, D.B., E. Padilla-Crespo, K.M. Ritalahti, S. Spring, H. Schmidt, M. Schmid, C. Lebrón, F.E. Löffler, and N.M. Lee.** 2009. Comparison of PCR and FISH for detection of bacteria in dechlorinating samples. *Partners in Environmental Technology Technical Symposium and Workshop.* December 1-3, 2009. Washington DC, USA.

44. **Meisinger, D. B., H. Schmidt, M. Schmid, C.A. Lebrón, F.E. Löffler, and N.M. Lee.** 2009. Unravelling the biodiversity in chloroorganic solvent contaminated aquifers. . *American Society for Microbiology 109th General Meeting.* May 17-21 2009, Philadelphia, PA, USA.

45. **Meisinger, D. B., R. Rossello-Mora, S. Spring, F.E. Löffler and N.M. Lee.** 2009. Insights into the culturability of specimens within the phylum Chloroflexi. *BioTech 2009: Isolation, Identification and Preservation of Microorganisms*. June 29-30, 2009, Zürich University of Applied Sciences, Wädenswil, Switzerland.

46. **Padilla-Crespo E., C. Cruz-García, L. Hung, K.M. Ritalahti, K.T. Konstantinidis, E.A. Edwards, and F. E. Löffler.** 2009. DNA microarrays elucidate new biomarkers implicated in reductive dechlorination. *American Society for Microbiology 109th General Meeting*. May 17-21, Philadelphia, PA, USA

47. **Padilla-Crespo E., T.L. Shearer, A. Massol-Deyá, E. Otero-Morales, K.M. Ritalahti and F.E. Löffler**. 2009. Detection of dechlorinating *Chloroflexi* in marine environments. *American Society of Limnology and Oceanography Aquatic Sciences Meeting*. January 25-30, 2009, Nice, France.

48. **Padilla, E., L.A. Hug, E.A. Edwards, and F.E. Löffler.** 2009. Microarrays for reductive dechlorination biomarker identification and *Dehalococcoides* monitoring, *SERDP/ESTCP Partners in Environmental Technology Technical Symposium & Workshop*. Washington, DC, USA.

49. **Pavlekovic, M., D.B. Meisinger, S. Spring, M. Schmid, I. Nijenhuis, H.H. Richnow, G. Diekert, C.A. Lebrón, F.E. Löffler, and N. M. Lee.** 2009. In Situ detection of dechlorinating bacteria including *Dehalobacter*, *Desulfitobacterium* and *Sulfurospirillum* spp. *American Society for Microbiology 109th General Meeting*. May 17-21 2009, Philadelphia, PA, USA

50. **Ritalahti, K.M., K.T. Konstandtinidis, and F.E. Löffler.** 2009. Insights into mutualistic interactions between free-living pleomorphic spirochetes (FLiPS) and obligate dechlorinators (*Dehalococcoides*). . *American Society for Microbiology 109th General Meeting*. May 17-21 2009, Philadelphia, PA, USA.

51. **Tang, S., A. Grostern, W. Chan, and E.A. Edwards. 2010**. Characterization of *Dehalobacter* cultures that reductively dechlorinate chlorinated ethanes and chloroform, *13th International Symposium on Microbial Ecology*. Seattle, WA, USA

2008

52. **Chan, W., A. Grostern, E.A. Edwards.** 2008. A cross-inhibition study of anaerobic trichloroethene and 1,1,1-trichloroethane degradation with the use of cell-free extracts. Abstract Q-015. *108th General Meeting of the American Society for Microbiology*, June 1-5, 2008, Boston, MA, USA

53. **Dumas, M.D., K.E. Fletcher, R.R. Helton, K.M. Ritalahti, F.E. Löffler, and K.E. Wommack. 2008**. Growth of *Dehalococcoides* sp. strain BAV1 in conjunction with a highly infective phage. Abstract N-288. *108th General Meeting of the American Society for Microbiology*. June 1-5, 2008, Boston, MA, USA.

54. **Grostern, A. and E.A. Edwards**. 2008. Enrichment of a *Dehalobacter* sp. that dichloroeliminates 1,2-DCA. Abstract Q-002. *108th General Meeting of the American Society for Microbiology*. June 1-5, 2008, Boston, MA, USA.

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